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Merging Microbial Electrochemical Systems with Conventional Reactor Designs for Treating Wastewater

Memoria presentada para optar al título de Doctor por la Universidad de Alcalá por :

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Cuando emprendas tu viaje hacia Itaca
debes rogar que el viaje sea largo,
rico en aventuras, lleno de conocimientos.
No has de temer ni a los lestrigones ni a los cíclopes,
ni la cólera del airado Poseidón.
Nunca tales monstruos hallarás en tu ruta
si tu pensamiento es elevado, si una exquisita
emoción penetra en tu alma y en tu cuerpo.
Los lestrigones y los cíclopes
y el feroz Poseidón no podrán encontrarte
si tú no los llevas ya dentro, en tu alma,
si tu alma no los conjura ante ti.
Debes rogar que el viaje sea largo,
que numerosas sean las mañanas de verano
en que con placer, felizmente
arribes a bahías nunca vistas;
Detente en los emporios de Fenicia
y adquiere hermosas mercancías,
madreperlas y coral, y ámbar y ébano,
perfúmenes deliciosos y diversos,
cuanto puedas invierte en voluptuosos y delicados perfumes;
visita muchas ciudades de Egipto
y con avidez aprende de sus sabios.

Ten siempre a Itaca en tu mente:
llegar allí es tu destino.
Mas no apresures tu viaje;
mejor que dure muchos años,
y que llegues, ya viejo, a la pequeña isla,
rico de cuanto habrás ganado en el camino,
sin esperar que Itaca te enriquezca.
Itaca te regaló un hermoso viaje.
Sin ella, jamás habrías partido;
mas no tiene otra cosa que ofrecerte.
Y si la encuentras pobre, Itaca no te ha engañado.
Y siendo ya tan viejo, con tanta experiencia,
sin duda sabrás ya qué significan las Itacas.

Konstantinos Kavafis. *Itaca*. Poesías completas, 1911.

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Summary

Summary

Microbial electrochemistry or electromicrobiology has emerged as a new subdiscipline of the biotechnology based on the study of the interactions between microbial living cells and electrodes. The catalytic properties of these microorganisms are very versatile and a diversity of fields can benefit from these systems known collectively as Microbial Electrochemical Technologies (METs). These technologies have emerged as novel systems that fill well within the recently recognized water-energy nexus by reason of their attractive applications in wastewater treatment and water desalination. However, the implementation of METs in real-world applications depends upon the resolution of microbial, technological and economical challenges. To date, METs have been understood as devices in which the catalysis is located at the electrode interface due to the need of microbial attachment forming a biofilm. The need of optimizing this interaction is the main challenge of the field, and has been mainly focused on improving the reactor and electrodes design, in addition to achieving better extracellular electron transfers mechanisms.

In this thesis, we have explored new scenarios and strategies for overcoming the technological bottlenecks of METs in the wastewater treatment applications. The work has been organized in 5 chapters, 3 of them being experimental.

Chapter 1 constitutes an introductory section to the microbial electrochemistry field with a description concerning the state of the art of the applying METs to wastewater treatment.

Fundamenetal studies of the microbe-electrode interaction and the catalytic process are essential for optimizing the performance of the bioelectrochemical systems. In this regard, *Geobacter sulfurreducens* is considered the model microorganism of choice performing direct extracellular electron transfer (DEET) in microbial electrochemistry and thus, is extensively used in proof-of-concept assays. This bacterium typically forms multi-layer biofilms on METs electrodes. However, *Geobacter*, in its natural habitat, is typically planktonic when respiring insoluble electron acceptors as iron oxides. The biofilm configuration limits the performance of the system due to the restriction of having the reaction occur at the electrode-biofilm interface. This presents problems associated with the activity of the cells within the biofilm.

In **Chapter 2**, we have designed a microbial electrochemical fluidized bed reactor (ME-FBR) with the aim of maximizing the superficial area of anode being

available for electroactive microorganisms, and of improving the kinetics of the catalysis by employing an environment with favorable mixing properties. This scenario was achieved by merging a classical fluidized reactor with a MET. We explored the interaction of *G. sulfurreducens* with a fluidized 3D-anode composed of glassy carbon microparticles that served as an electron acceptor for these bacteria. Interestingly, in this situation, the bacteria-electrode interaction was occurred in motion and under a planktonic state of both the anode and the bacteria. Actually, to our delight, this interaction nicely supported the microbial growth, a fact that **suggests a new paradigm in the direct extracelular electron transfer in METs**. Our results have revealed a **novel mode to culture electroactive bacteria where every single cell in the medium could be instantaneously wired to a fluid-like electrode.** Culturing *Geobacter* cells respiring a fluid-like anode displayed **a phenotype that was able to respire insoluble iron oxide faster than did cells previously grown with a soluble electron acceptor**. This implies that the cells grown respiring the fluidized bed anode shared common traits with *G. sulfurreducens* cells growing in their natural habitat, where iron oxides, their natural electron acceptor, are dispersed.

In **Chapter 3** we used this ME-FBR to treat real wastewater. In this study we worked with a mixed culture and with a fluidized bed made of porous and hydrophilic microparticles of activated carbon in order to promote a rapid microbial colonization of them. This modification allowed us to operate the system in a continuous mode avoiding the possibility of washing-out the suspended bacteria. In parallel, we ran a similar system but with a fluidized bed fabricated of a non-conductive material so as to compare the electrogenic treatment versus a classical anaerobic digestion in a fluidized bed configuration. Here, we observed that **the ME-FBR was able to remove up to 95 % of the chemical oxygen demand (COD). In fact, this reactor outperformed the anaerobic biolite-ME-FBR reactor** and the differences were more marked as the organic loading rate was increased. We observed larger levels of volatile fatty acids (mostly acetate) in the non-electrogenic treatment, indicating that the methanogenesis was the main rate-limiting step in that system. In contrast, trace levels of acids were found in the ME-FBR indicating a rapid consumption of acids coupled to current generation. In addition, the polarized particles from the ME-FBR showed a more extended microbial colonization as compared to the biolite particles. *Geobacter* species were highly enriched in the polarized particles, being located at the innermost layers of the biofilm. This results serve as a **validation of using a ME-FBR for efficiently removing the organic matter in industrial wastewaters**.

METs have been shown to stimulate the organic matter degradation in many kinds of wastewaters by supplying to electroactive microorganisms with an anode as a final electron acceptor. However, the limitations of METs for completely treating wastewaters suggest the need for supporting these systems with a complementary technology able to remove nutrients and/or suspended matter. In **Chapter 4** we have addressed this problem by adopting different strategies for eliminating the nutrients of wastewaters that could be coupled to a ME-FBR treatment.

In the first part of Chapter 4, we propose a bioelectrochemical treatment in a configuration that simulates a classical 2-chamber activated sludge design for removing nitrogen from low COD effluents. With this hybrid system it was possible to **remove up to 81 % of the nitrogen without any external aeration and with a cathode serving as electron donor** for denitrifying microorganisms. Low quantities of sludge were produced throughout the entire experimental period. The nitrification process was performed under a micro-aerobic environment and without detecting in this reaction mediation of the anode. We propose adapting METs to previously constructed reactors, integrating these hybrid reactors into urban wastewater treatment plants with minimal infrastructure costs beyond the installation of electrodes.

In the second part of Chapter 4, we have developed a unit operation based on integrating an electrocoagulation (EC) step as a pre-treatment, followed by a ME-FBR as an organic matter-removing step in brewery wastewater. In our proposal, removal of nutrients and insoluble matter is isolated from the soluble organic matter biodegradation stage. With the **EC pre-treatment we were able to remove most of the nutrients and suspended solids** that are not esaily removed in biological anaerobic reactors. By varying parameters as the applied current density or reaction time in the EC, we could tune the nutrients concentration in the effluent. The soluble organic matter fraction of the effluent from the EC could then be successfully eliminated in the ME-FBR. In this work, we demonstrate that by **merging two kinds of electrochemical techniques, such as EC and ME-FBR, one can achieve an effective strategy for completely treating wastewater from breweries but likely other wastewaters as well**.

Finally, in Chapter 5 we present a general discussion, conclusions and future work based on our experimental results. The style of this section has been developed in the framework of a question-answer session. In this thesis we present new scenarios for treating wastewater based on employing classical reactor designs merged with microbial electrochemical technologies. We believe that by using these

kinds of configurations, the process of scaling-up METs could be more straightforward. Economically, this strategy would allow one to easily scale-up prototypes to real world conditions in order to assess novel technologies as METs that otherwise are confined at the lab scale.

Resumen

La electroquímica microbiana o electromicrobiología ha surgido como una nueva subdisciplina de la biotecnología basada en el estudio de las interacciones entre microorganismos y electrodos. Las propiedades catalíticas de estos microorganismos son muy versátiles y una diversidad de campos pueden beneficiarse de ellas a través del desarrollo de las tecnologías electroquímicas microbianas (MET). Los dispositivos empleados en estas tecnologías se han convertido en sistemas novedosos que reflejan perfectamente el nexo agua-energía a causa de sus aplicaciones atractivas en el tratamiento de aguas residuales y la desalinización del agua. Sin embargo, la aplicación de las METs a escala real depende de la resolución de desafíos microbiológicos, tecnológicos y económicos. Hasta el momento, las METs se han entendido como sistemas en los que la catálisis se encuentra localizada en la interfaz del electrodo debido a la necesidad de adhesión microbiana formando un biofilm sobre él. La optimización de esta interacción es el principal reto del campo y se centra principalmente en la mejora del diseño del reactor y de los electrodos y en la optimización de los mecanismos de transferencia de electrónica extracelulares.

En esta tesis se han explorado nuevos escenarios y estrategias para superar los cuellos de botella tecnológicos de las METs en su aplicación para el tratamiento de aguas residuales. El trabajo se ha organizado en 5 capítulos, 3 de ellos experimentales. El Capítulo 1 constituye una sección de introducción al campo de la electroquímica microbiana con un estado del arte de la aplicación de las METs en el campo del tratamiento de aguas residuales.

El estudio de los fundamentos de la interacción bacteria-electrodo y del proceso catalítico son esenciales para la optimización del rendimiento de los sistemas bioelectroquímicos. *Geobacter sulfurreducens* se considera el microorganismo modelo de transferencia electrónica directa extracelular (DEET) en la electroquímica microbiana y, por tanto, se utiliza ampliamente en los ensayos de prueba de concepto. Esta bacteria forma típicamente biopelículas de múltiples capas sobre los electrodos de las METs. Sin embargo, *Geobacter*, en su hábitat natural, se encuentra en estado planctónico al respirar aceptores insolubles de electrones como los óxidos de hierro. La configuración de biopelícula limita el rendimiento del sistema debido a la restricción de la reacción a la interfase electrodo-biofilm y presenta problemas asociados con la actividad de las células dentro de la biopelícula. Con el objetivo de maximizar el área superficial de ánodo disponible para los microorganismos electroactivos, y de mejorar la cinética de la

catálisis empleando un entorno con buenas propiedades de mezcla, en el Capítulo 2 diseñamos un reactor de lecho fluidizado electroquímico microbiana (ME-FBR). Este escenario se logró mediante la fusión de un reactor fluidizado clásico con una MET. Estudiamos la interacción de *G. sulfurreducens* con un ánodo de 3 dimensiones fluidizado compuesto por micropartículas de carbón vítreo que servía como aceptor final de electrones para esta bacteria. Curiosamente, en esta situación, la interacción bacteria-electrodo se realizó en movimiento y bajo el estado planctónico de ambos elementos. De hecho, permitió el crecimiento microbiano, lo cual **supone un nuevo paradigma en la transferencia de electrones directa dentro del campo de las METs**. Nuestros estudios han revelado un **modo nuevo de cultivar bacterias electrogénicas en el cual cada célula, de forma individual, está trasnsitoria y directamente conectada con una partícula de ánodo fluidizado**. Estas células de *Geobacter* mostraron un **fenotipo capaz de respirar óxido de hierro insoluble de formas más efectiva que las células cultivadas previamente con un aceptor de electrones soluble**. Por tanto, las células cultivadas respirando el ánodo fluidizado podrían compartir estrategias comunes con células de *G. sulfurreducens* en su hábitat natural, donde los óxidos de hierro, su aceptor de electrones natural, se encuentran dispersos.

Tras demostrar que un ánodo fluidizado podía ser utilizado como un elemento de descarga de electrones para *Geobacter* y que, además, podía actuar como aceptor de electrones para oxidar acetato durante un período prolongado de tiempo, se procedió a estudiar, en el Capítulo 3, el tratamiento de un agua residual real en un ME-FBR. En estos ensayos se trabajó con un cultivo mixto y con un lecho de micropartículas porosas e hidrófilas de carbón activado con el fin de promover la colonización de las partículas. De esta forma, fuimos capaces de operar el sistema en modo continuo evitando la posibilidad de un lavado de biomasa. En paralelo, operamos un sistema fluidizado con un lecho de un material no conductor para poder comparar el tratamiento electrogénico frente a una digestión anaeróbica clásica en una configuración de lecho fluidizado. Se observó que el ME-FBR fue capaz de eliminar hasta el 95% de la demanda química de oxígeno (COD). De hecho, superó al reactor con lecho de no conductor y las diferencias se hicieron más notables a medida que se aumentó la velocidad de carga orgánica. Se encontraron mayores niveles de ácidos grasos volátiles (en su mayoría acetato) en el tratamiento no electrogénico, indicando que la metanogénesis era la principal etapa limitante en ese sistema. En contraste, se encontraron niveles de traza de ácidos en el ME-FBR, lo cual indicó un consumo rápido de ácidos acoplados a la generación de corriente. Se encontró una colonización microbiana mayor en las partículas polarizadas del

ME-FBR en comparación con las partículas no conductoras. Además, la biopelícula formada sobre las partículas polarizadas se enriqueció en especies de *Geobacter*, las cuales se localizaron principalmente en las capas más internas de la biopelícula. Con los resultados de este estudio, se **validó el uso de un ME-FBR para eliminar eficazmente la materia orgánica de aguas residuales industriales**.

Las METs han demostrado la estimulación de la degradación de la materia orgánica de muchos tipos de aguas residuales a través del suministro a los microorganismos electroactivos de un ánodo como aceptor de electrones final. Sin embargo, las limitaciones de las METs para tratar aguas residuales de forma completa sugieren la necesidad de apoyar estos sistemas con una tecnología complementaria capaz de eliminar los nutrientes y/o materia en suspensión. En este contexto, en el Capítulo 4 hemos abordado este aspecto estudiando diferentes estrategias para la eliminación de nutrientes de las aguas residuales que podrían ser acopladas a un tratamiento en un ME-FBR.

En la primera parte de este capítulo, se propone un post-tratamiento bioelectroquímico en una configuración que simula un diseño clásico de lodos activados de 2 cámaras para eliminar el nitrógeno de efluentes con baja materia orgánica. Con este sistema híbrido **fue posible eliminar hasta el 81% del nitrógeno sin aporte de aireación externa y con un cátodo que servía como donador de electrones** para microorganismos desnitrificantes. Se produjeron cantidades bajas de lodo a lo largo de todo el período experimental. El proceso de nitrificación se realizó bajo un ambiente micro-aerobio y sin mediación del ánodo. Con este estudio, **proponemos la adaptación de METs a reactores ya construidos e integrarlos en las plantas de tratamiento de aguas residuales**, sin costes de infraestructura adicionales más allá de la instalación de los electrodos.

En la segunda parte del Capítulo 4 desarrollamos un proceso conjunto, basado en la integración de una etapa de electrocoagulación (EC) como un pre-tratamiento, y de un ME-FBR como etapa para la eliminación de materia-orgánica. En nuestra propuesta, la eliminación de nutrientes y de materia insoluble se separa de la fase de biodegradación de materia orgánica soluble. Con el pre-tratamiento EC fuimos capaces de eliminar la mayor parte de los nutrientes y sólidos en suspensión que no son capaces de eliminar los reactores anaerobios biológicos. Mediante la variación de parámetros como la densidad de corriente aplicada o el tiempo de reacción en la EC, se puede controlar la concentración de nutrientes en el efluente. La fracción de materia orgánica soluble del efluente de la EC fue eliminada con éxito en un ME-FBR. En este trabajo se demuestra que **la integración de dos tipos de**

técnicas electroquímicas, como la EC y un ME-FBR, resulta en una estrategia eficaz para el tratamiento completo de aguas residuales industriales.

Por último, en el Capítulo 5 se presenta una discusión general, conclusiones y perspectivas futuras, basado en nuestros resultados experimentales. El estilo de esta sección ha sido desarrollado a modo de pregunta-respuesta. En esta tesis presentamos nuevos escenarios para el tratamiento de aguas residuales basados en el empleo de diseños de reactores clásicos fusionados con tecnologías electroquímicas microbianas. Creemos que, mediante el uso de este tipo de configuraciones, el proceso de escalado de las METs podría ser más sencillo y directo. Económicamente, permitiría operar prototipos fáciles de instalar a gran escala para evaluar tecnologías novedosas como las METs, que, de otro modo, estarían estancadas en escala laboratorio.

CHAPTER 1: Introduction, objectives and research framework

Introduction

1.1 Microbial Electrochemistry: Fundaments

1.1.1 The Origins Of Microbial Extracellular Electron Transfer (EET)

The phenomenon of microbial electrochemistry is based on the capacity of certain microorganisms to exchange electrons with a terminal electron acceptor (TEA) or electron donor (ED) characterized for being a conductive and insoluble form. The first evidences of this process were observed in marine sediments, where the presence of electrode-reducing microorganisms able to transfer the electrons resulting from their metabolism to an electrode, generating then an electric current, was firstly reported (Reimers *et al.*, 2001; Bond *et al.*, 2002). These microorganisms were able to conserve energy to support their growth by oxidizing organic compounds in the marine sediments with an electrode serving as the sole TEA. This novel mode of metabolism has given rise to an emergent research field based on the activity of these microorganisms, so-called electrogens, electroactive bacteria or exoelectrogens.

This remarkable capacity of some microorganisms for interacting with electrodes is still a surprise and not well-understood process among the scientific community. One of the features that share most of the reported electroactive bacteria is their natural capacity for reducing metals such as insoluble iron forms. Fe (III) is the most abundant electron acceptor available in soils and sediments, the natural habitat of *G. sulfurreducens* (Lovley *et al.*, 2011). This Gram-negative specie is known as the model microorganism in the microbial electrochemistry field. Fe (III) is the natural TEA of *Geobacter* species in the presence of acetate, hydrogen or lactate as electron donors (Caccavo *et al.*, 1994; Speers and Reguera, 2012).

One of the first hypotheses for explaining the microbial electron-exchanging capacity with electrodes was that the metal-reducing evolutionary mechanisms of those microorganisms resulted to be effective for reducing electrodes as well (Lovley, 2012). However, the mechanisms for reducing metals and electrodes seem to differ. For instance, when *Geobacter* species are grown with Fe (III) oxides, they express flagella that use as a motility element for the search of the next source of Fe (III) (Childers *et al.*, 2002). In contrast, when *Geobacter* is cultured in an electrochemical system, they permanently attach to the electrodes forming a biofilm (Bond and Lovley, 2003). Another aspect that questions that hypotheses is the fact that not all the metal-reducing microorganisms are able to respire electrodes. For

instance, *Pelobacter carbinolicus*, which reduces Fe(III) oxides, does not have the ability for transfering electrons to anodes (Richter *et al.*, 2007).

Another hypothesis is the so-called theory of geobatteries, giant electrochemical cells naturally formed. Geobatteries are graphite deposits formed in the subsurface that can electrically connect regions of different redox potential such as anoxic and aerobic environments (Bigalke and Grabner, 1997). This kind of geobatteries would constitute a long-term electron acceptor or electron donor for the surrounding microbes and have features in common with the electrodes acting as anodes or cathodes (Leung and Xuan, 2015).

The most recent hypothesis relates this capacity for EET to electrically conductive materials with the ability of microorganisms to directly use another cell as TEA, establishing an electrical contact between two microorganisms. This is called direct interspecies electron transfer (DIET) and is a kind of interspecies electron transfer (IET), which enables a diversity of microbial communities to gain energy from reactions that no one microbe can catalyze (Shrestha and Rotaru, 2014). It is a mechanism for exchanging electrons during syntrophic metabolism. The capacity for DIET via outer membrane elements or via nanowires (Gorby *et al.*, 2006) is hypothesized to be what confers electroactive bacteria their ability to interact with electrodes. For instance, the metal-reducing microorganism *Pelobacter carbinolicus*, which is in the same phylogenetic family as *G. metallireducens*, is incapable of performing DIET and reducing anodes (Rotaru *et al.*, 2012). In another study, it was seen that only high current density producing *Geobacter* species could interact via DIET with *Methanosarcina barkeri,* what suggests that there could be correspondence between the ability to produce high currents and the ability to grow syntrophically (Rotaru *et al.*, 2015).

A similar strategy of transporting electrodes for resource competition has been observed via living micro-cables over long distances in the form of long filamentous bacteria of the Desulfobulbaceae family (Pfeffer *et al.*, 2012). The electrons generated by sulphide oxidation in cells at one end can be conducted through internal, insulated wires to cells at the other end, where oxygen is reduced (Figure 1-1) The reaction is called electrogenic sulphur oxidation. This is an ability to separate soluble electron acceptors and donors in space (over centimeter distances) and a strategy for energy conservation in marine, freshwater, and salt-marsh sediments (Larsen *et al.*, 2015; Risgaard-Petersen *et al.*, 2015; Schauer *et al.*, 2014).

Figure 1-1: A: Cells of *Shewenella oneidensis* connected by microbial nanowires, composed of pilin protein. From Gorby *et al*, 2006. B: Filamentous Desulfobulbaceae cells (yellow) identified by fluorescence in situ hybridization forming a micro-cable. From Pfeffer *et al*, 2012. How do microbes perform extracellular electron transfer?

1.1.2 How Do Microbes Perform Extracellular Electron Transfer?

The respiration is a process that converts the redox potential gradient between two chemical compounds into a biological form of energy, generally ATP (adenosine triphosphate). Some microorganisms utilize a wide range of soluble reduced compounds as electron donors (*e.g.*, acetate, lactate, H_2, CH_4, sulfides, ammonia…) and soluble oxidized forms as electron acceptors (*e.g.*, nitrate, sulfate, O_2 or CO_2). Other microorganisms utilize solid substrates for their respiration, such as minerals or electrodes. Another kind of respiratory substrates are insoluble forms unable to pass the outer membrane of Gram-negative bacteria, such as the quinones present in humic substances (Richter *et al.*, 2012). The two last ones are specifically called EET, which is a type of microbial respiration that involves the electron transfer between microbial cells and extracellular materials (see Figure 1-2). Special molecular mechanisms are required for EET because microorganisms cannot incorporate such insoluble materials into their cells and thus the electrons need to go through periplasm and over the outer membrane.

The microbial EET is a natural process of notable importance considering that iron is the most abundant redox-active metal in today's Earth's crust. It can contribute to the organic matter oxidation in a wide diversity of anaerobic environments.

Direct Extracellular Electron Transfer (DEET)

DEET requires a physical contact between the microorganism and the electrode, usually attached forming a biofilm on the electrode surface (Figure 1-2.A and B). This mechanism involves transmembrane redox-active proteins as *c*-type

cytochromes (Allen *et al.*, 2009; Busalmen *et al.*, 2008). The DEET via outer membrane cytochromes requires the physical contact of the bacterial cell to the electrode, with the consequence that only bacteria in the first monolayer at the anode surface are electrochemically active. This causes a limitation of the catalysis by the maximum cell density in this bacterial monolayer. However, it has been demonstrated that some species have developed nanowires or pili to reach and utilize distant insoluble electron acceptors or to interconnect inners layers in the biofilm. This kind of strategy has been observed for *Geobacter* and *Shewanella* species (Reguera *et al.*, 2005; Gorby *et al.*, 2006).

Another example of DEET is when a cell uses another cell as TEA via DIET. This phenomena was firstly described in *G. metallireducens* and *G. sulfurreducens* co-cultures, growing them in a medium with ethanol as electron donor (not utilized by the second specie) and fumarate as electron acceptor (not utilized by the fisrt specie) (Summers *et al.*, 2010). The aggregates formed were actually electrically conductive, like anode biofilms (Malvankar *et al.*, 2011). Later, it was also seen that some species of methanogens like *Methanosarcina barkeri* were capable of performing DIET in co-cultures with *Geobacter* species (Rotaru *et al.*, 2014, 2015), a fact that might be relevant in the methane production in anaerobic digesters.

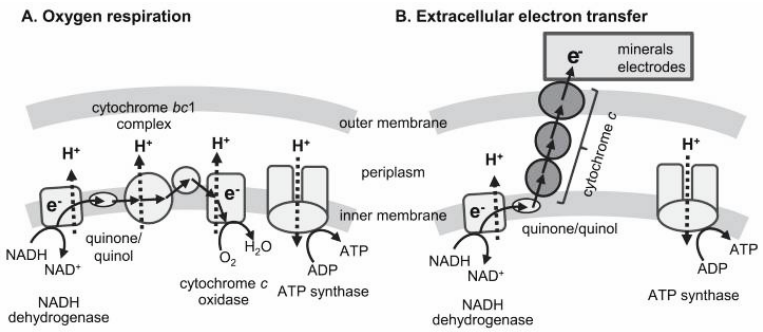

Figure 1-2: Microbial respiration and electron transfer to a: A: soluble electron acceptor as oxygen and B: to a solid substrate as a mineral. From Kato S., 2015. (Kato, 2015).

DIET can also take place with a mineral as mediator, a process in which different species use as conduits of electrons nano-mineral particles or conductive surfaces such as activated carbon granules or biochar (Kato *et al.*, 2012; Liu *et al.*, 2012). The contact with these extracellular solids is still performed via DEET. It has been reported the ability of conductive minerals to simultaneously enhance the growth of *Geobacter* species and methane production (Cruz Viggi *et al.*, 2014; Li *et*

al., 2015; Shrestha and Rotaru, 2014). This suggests that co-aggregation of *Geobacter* species and methanogens may be a common phenomenon in these methanogenic environments.

Mediated Extracellular Electron Transfer (MEET) To Insoluble Substrates

In this type of mechanism, low-molecular compounds, referred to as electron mediators, act as the electron carriers between microbial cells and solid materials (Figure 1-3.C). Some microorganisms can naturally synthesize and excrete endogenous redox-active molecules that function as electron mediators, such as flavins or phenazine compounds. Likewise, these mediators can be external agents, artificially added to the media or present in natural environments as humic substances. These electron shuttles may be reduced by outer-surface redox-active molecules as *c*-type cytochromes (Kotloski and Gralnick, 2013; Voordeckers *et al.*, 2010).

Interspecies electron transfer can occur as well through a mediated process. The electrons travel from one substrate to another through redox reactions mediated by microbial metabolism. The most common case is through H_2: i.e. electron-donating microorganisms reduce protons to H_2 and another one, as a methanogen, oxidize this H_2 reducing carbon dioxide to methane. Other intermediate metabolites such as formate and acetate can also function as electron carriers in the same was as H_2 (Shrestha and Rotaru, 2014).

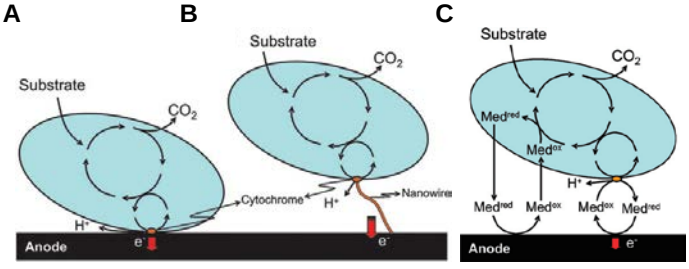

Figure 1-3: A. DEET to electrodes via membrane bound cytochromes. B. DET via electronically conducting nanowire. 2. MEET via secondary metabolites. From Schröder (2007).

1.1.3 *Geobacter* As Model Bacteria In Microbial Electrochemistry

The model electroactive microorganism and best studied is *Geobacter sulfurreducens*. It was indeed the first microorganism described for using iron oxide

as TEA (Lovley *et al.*, 1987). It is an anaerobic (non obligated), non-fermentative, rod-shaped Gram-negative bacterium. It can utilize a variety of electron donors (acetate, hydrogen, lactate) and of electron acceptors (ferric iron, manganese oxides, fumarate, uranium, elemental sulphur, cathodes…) for respiration (dissimilatory reactions), but fewer substrates (like acetate) as carbon sources (assimilation reactions) (Bond and Lovley, 2003; Caccavo *et al.*, 1994; Speers and Reguera, 2012). Acetate is the preferred electron donor for *Geobacter*, and is the central intermediate in the anaerobic degradation of organic matter in sedimentary environments. In addition, it is the end-product of the acetogenic phase in the anaerobic digestion process, and its presence is related to biogas production (Henze, 2008). It should be noted that *Geobacter* electroactive biofilms can oxidize acetate coupled to the reduction of an anode by utilizing more than 96% of the electrons contained in this substrate for respiration (Bond and Lovley, 2003; Nevin *et al.*, 2008).

The genome of *G. sulfurreducens* has been decoded and contains 111 putative genes coding for *c*-type cytochromes, many of them containing multiheme groups (Methe *et al.*, 2003). Because of this vast heme-network (Figure 1-4) distributed from the periplasmic space (Lloyd *et al.*, 2003) to the outermost membrane (Inoue *et al.*, 2010; Mehta *et al.*, 2005), pili (Leang *et al.*, 2010), and even extracellular matrix (Rollefson *et al.*, 2011), *Geobacter* is able to perform EET to insoluble electron acceptors efficiently. Actually, limiting the production of *c*-type cytochromes in *Geobacter* eliminates its EET capacity, producing an inhibition on the current generation in anodes (Estevez-Canales *et al.*, 2014). However, the exact role of each cytochrome and the exact pathway of EET remain unknown, and some of the elements involved seem to have different roles in the respiration of iron oxides than in electrodes.

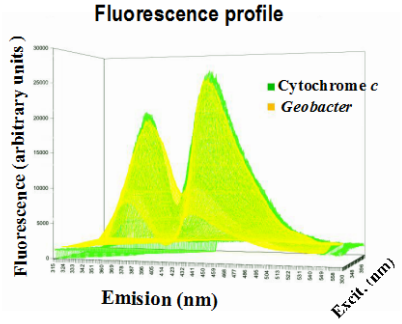

Figure 1-4: Fluorescence profiles of a pure suspension of cytochrome *c*, and of a suspension of *G.sulfurreducens* cells. From Esteve-Núñez *et at.* (2008).

Besides acting as electron mediator in the EET to electrodes, the network of cytochromes in *Geobacter* planktonic cells can also function as a short-term sink for the electrons from the acetate metabolism when extracellular electron acceptors are not available (Figure 1-5). This is the so-called capacitor effect (iron lungs) (Esteve-Núñez *et al.*, 2008; Lovley, 2008). The same effect has been seen for electroactive biofilms on anodes. When the polarization of an anode colonized with an electroactive biofilm is interrupted and remains at open circuit potential (OCP) (no electron acceptor available), the anode potential rapidly decreases to negative values down to -0.34 V (vs Ag/AgCl reference electrode (RE)) (Schrott *et al.*, 2011). This change is interpreted as the consequence of the accumulation of reduced electroactive species at the electrode surface. This redox species are the outer membrare cytochromes that are contacting the electrode, as it has been previously reported (Busalmen *et al.*, 2008). When the electrode is reconnected, acting thereby as electron acceptor, there is a discharge of electrons accumulated in the biofilm during the disconnection time. The electron storage capacity of *Geobacter* electroactive biofilms has actually been reported as comparable to that of synthetic supercapacitors with low self-discharge rates (Schrott *et al.*, 2011; Liu *et al.*, 2011; Malvankar *et al.*, 2012).

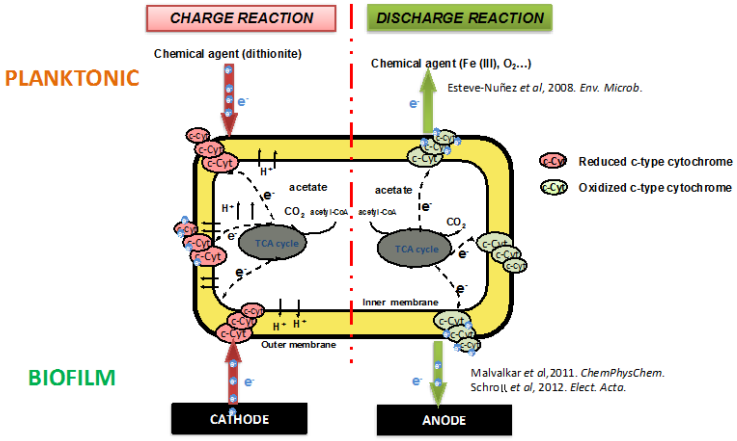

Figure 1-5: Electron storage capacity of *Geobacter* cytochrome network for planktonic cells and for biofilms and the charging and discharging methods reported for each of them.

Geobacter Electroactive Biofilms: Paradigm And Alternatives

The biofilm-based physiology is the paradigm on electricity-harvesting bacteria. It is the grown mode in which *Geobacter* has been consensually considered

as the electroactive model bacteria. This biofilm architecture can reach thicknesses of *ca.* 100 µm, and cells without direct contact with the electrode can perform EET via electrically conductive pili. However, once biofilms are thicker than 60-70 µm, the accumulation of cells in the outermost layers does not contribute to current production (Schrott *et al.*, 2014).

In contrast to the physical accommodation in bioelectrochemical systems, *Geobacter* has performed DEET in a cellular planktonic state for millions of years in the subsurface (Childers *et al.*, 2002; Holmes *et al.*, 2002). Planktonic cells of *Geobacter* cultured in freshwater medium show a poor electroactive phenotype. However, they are highly electroactive when cultured under soluble electron acceptor limitation in a chemostat under continuous culture (Esteve-Núñez *et al.*, 2011). These cells have been named as *plug-and-play* cells due to their ability to rapidly exchange electrons with an electrode and coupling this process with their metabolism. Using this kind of inoculum in microbial electrochemical cells allows reducing the starting-up periods in these systems (Borjas *et al.*, 2015).

Another example of *Geobacter* planktonic electroactive phenotype is the one recently achieved by Borjas *et al* by growing this bacterium in a salt-supplemented media (Borjas Z., 2016). These culturing conditions provoked an osmotic response in the cell under which the cells synthesized extracellular polymeric substances (EPS) that acted as an electroactive network.

Despite the great research carried out regarding the elucidation and optimization of the interaction bacteria-electrode, it still represents a challenge key for establishing new frontiers and practical applications in the field of electromicrobiology.

1.2 Microbial Electrochemical Technologies: A Versatile Platform For Environmental Applications

Microbial electrochemistry is the discipline focused on the interactions between living microbial cells and conductive materials (electrodes). Although the bacterial capacity to couple the oxidation of organic matter to the reduction of electrodes was known since the origins of the XX century (Potter, 1911), it took 50 years to develop the first Microbial Fuel Cell device (MFC) (Berk and Canfield, 1964; Hees, 1965). However, it was not considered a real technological alternative until the beginning of XXI century. Indeed, it was in 2001 when the MFCs field went through a revolution whose origin was the key research from Reimers *et al.* (Reimers *et al.*,

2001). They demonstrated that electric energy could be harvested from the natural voltage gradient generated between the anoxic zone and the overlaying oxygenic seawater in marine sediments. In addition, it was seen that this gradient was due to the presence of electroactive microorganisms. This was the real discovery that launched the beginning of a new MFC field.

Since then, the capacity of extracellular electron transfer has been demonstrated for a wide variety of microorganisms: *Geoalkalibacter* spp. (Badalamenti *et al.*, 2013; Carmona-Martínez *et al.*, 2013a), *Geobacter* spp. (Bond and Lovley, 2003; Strycharz *et al.*, 2008), *Shewanella spp*. (Bretschger *et al.*, 2007; Carmona-Martínez *et al.*, 2013b), autotrophic microosganisms (Marshall *et al.*, 2012; Puig *et al.*, 2011a), methanogenic spp. (Villano *et al.*, 2010), etc. In addition, for a long list of metabolic reactions, as it will be shown in the next sections. Because of this, a large number of environmental applications have been developed based on the microbial catalysis of an electrode reaction. Any technology based on a microbial biocatalyst that exchanges electrons with an electrode is known as a Microbial Electrochemical Technology (METs). The most common classification of METs is based on the electrochemical operating mode employed. However, the increasing appearance of new applications has originated a new terminology that names the type of METs with regard to its use. In this section, it will be reviewed all these kinds of bioelectrochemical systems folliwing these two types of classification.

1.2.1 Classification Based On The Electrochemical Operation Mode

Microbial Fuel Cells (MFCs)

MFCs are devices that use bacteria as the catalysts to oxidize organic and inorganic matter and generate current (Logan, 2008). The basic design of a MFC consists of two chambers (anodic and cathodic) separated by an ion exchange membrane. In the anodic chamber, the organic matter (electron donor) is oxidized by bacteria, resulting CO_2, electrons and protons as by-products. These electrons are transferred from the anode to the cathode by an external electric circuit, while protons are transported to the cathodic chamber across the ion exchange membrane by a concentration gradient. In the cathodic chamber, oxygen (the most common electron acceptor) accepts those electrons and, in combination with the protons, it is reduced to water on the cathode surface (Figure 1-6.A). Microorganisms can also be cultured in the cathodic camera and catalyze a reduction reaction by using this electrode as electron source for their metabolism. As compared to an abiotic cathode, the biocathode can reduce a larger variety of compounds as oxygen,

nitrate, protons to form H_2, etc. Compared to traditional chemical fuel cells, the MFCs use low-cost and self-sustaining microorganisms to oxidize organic and inorganic electron donors, mainly waste materials, and transfer electrons to the anode electrode.

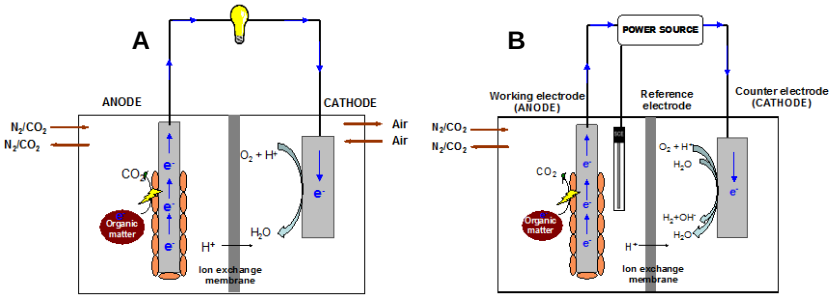

Figure 1-6: A: Schematic of a 2-chamber MFC with an anionic membrane separator. B: Schematic of a MEC configuration of 3 electrodes in which the anode is the working electrode.

Microbial Electrolysis Cells (MECs)

If electrical power is used to enhance the potential difference between the anode and the cathode of a MET, either to enable or to increase the rate of the electrode reactions, the system is called a MEC. The energy can be provided by a power source or a potentiostat depending on the selected mode of operation: galvanostatic mode (current flow is fixed) or at potentiostatic mode (the potential difference between two electrodes is fixed). In this latter case, if we want to maintain the potential of one of the electrodes under a selected value, we need to work, close to the so-called working electrode (WE), with a reference electrode (RE) in a 3-electrode configuration (see Figure 1-6.B). The other electrode is called counter or auxiliar electrode (AE) and its potential is dependent upon the current flow circulating through the system. This is one of the most extended configurations since it allows controlling the anodic or cathodic reactions, which is crucial for the study of the microbial-electrode interaction Figure 1-6.B shows a schematic of a 2-chamber MEC in which the potential of the anode is controlled and the organic matter oxidation is being performed at this WE. Although classical MFCs and MECs design is associated to this 2-chambered configuration, it is also possible to operate in a 1-chamber mode. In this configuration, WE and AE share the same electrolyte, eliminating the need of an ion exchange membrane. This reduces the costs associated with the use of this material but also notably reduces the ohmic

resistance of the system, which can improve current densities or decrease the energy demand of the external power supply (Logan, 2008).

Microbial Electrochemical Snorkel

This configuration is actually a short-circuited MFC. A short-circuited MFC provides the highest currents, meaning that it ensures the highest rate for organic matter oxidation. In the microbial electrochemical snorkel, one of the sides of an electrode plays the role of an anode and the other side the role of a cathode (Erable *et al.*, 2011). In theory, the anodic part should be exposed to anaerobic conditions and develop an electroactive biofilm over it, while a catalyst and/or an electroactive biofilm should form on the cathodic part exposed to the aerobic zone, as can be seen in Figure 1-7. The redox potential difference between both environments is the driving force of the electrons that circulate through the conductive material. The goal of this configuration is to maximize the pollutants removal, as is as been reported before i.e. for petroleum hydrocarbons (Cruz Viggi *et al.*, 2015), and not to harvest a current flow. Consequently, the system does not require complex electrochemical reactors with ionic exchange membranes or any other kind of separators. The design can be simplified to two connected electrodes, or even a single piece of conductive material.

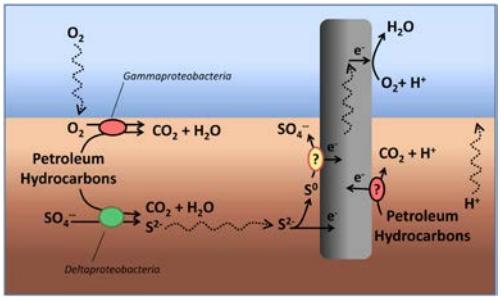

Figure 1-7: Schematic of a MES applied to biorremediatin of a polluted soil with petroleum hydrocarbons. From Cruz Viggi *et al.* (2015).

1.2.2 Prototypes Of METs And Applications

While many existing environmental technologies have only one or two functions, the METs platform is so flexible that dozens of them have been discovered. The most relevant ones are the direct power generation (MFCs) (Capodaglio *et al.*, 2013; Liu and Logan, 2004; Rabaey and Verstraete, 2005), production of H_2 (MECs) (Call and Logan, 2008; Logan *et al.*, 2008), microbial

electrosynthesis (Rabaey and Rozendal, 2010; Rabaey *et al.*, 2011), water desalination (microbial desalination cells, MDCs) (Cao *et al.*, 2009; Jacobson *et al.*, 2011), and even microbial electroremediating cells (MERCs) for restoring polluted environments (Rodrigo *et al.*, 2014). In this section, it will be briefly describe the fundaments of those applications, its possibilities and the future perspectives of the field.

Power Generation In MFC

MFC have shown their great potential as a technology for sustainable bioenergy production due to their ability to generate electricity mainly from wastewater while simultaneously treating it without the need of external aeration (Logan and Regan, 2006). Apart from fuels as urban wastewaters, electricity generation has also been demonstrated with domestic, food processing, and animal wastewaters (Angosto *et al.*, 2015; Kelly and He, 2014a; Rozendal *et al.*, 2008). The success of these devices relies on maximizing the power output of the system, which is achieved by minimizing the losses that reduce the potential difference between the anode and the cathode. The theoretical potential difference (maximum energy gain) between the biological standard potential ($E^{o'}$ [V vs standard hydrogen electrode (SHE)], 25 ºC, 1 atm, pH=7, ionic strength of 0.25 M)[1] of the terminal metabolic electron donor NADH (the intermediate electron carrier in microbial respiration) and the terminal electron acceptor oxygen is 1.14 V (+0.820 V - (-0.320 V)) (ec. [1] and ec. [2]).

$$NAD^+ + H^+ + 2e^- \rightarrow NADH \quad E^{o'}=0.320\ V \qquad [1]$$

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \quad E^{o'}=0.820\ V \qquad [2]$$

However, due to the activation, concentration and ohmic losses, this value is usually no larger than +0.51 V in MFCs (Schröder, 2007). Therefore, the efforts of researchers are currently focused on improving the kinetic rates, enhancing the coulombic efficiencies (CE) and optimizing the configurations for scaling-up (electrode materials, electrodes separation, minimization of internal resistance and optimization of the reactor design). Several designs of MFCs have been developed: two-chamber (Larrosa *et al.*, 2009), single-chamber (Liu and Logan, 2004) (Liu *et al.*, 2005a), upflow (He *et al.*, 2006), flat (Min and Logan, 2004), and tubular (Fradler *et al.*, 2014a). Among the different types of MFCs that have been developed, the air

[1] *The standard electrode potential of Ag/AgCl (in saturated KCl) against SHE is +0.2 V at 25 ºC.*

cathode MFC is the most likely configuration to be scaled up for wastewater treatment due to its high power output, simple structure, and relatively low cost.

So far, due to the low energy production in MFCs compared with other removable energy sources, its use seems to be more adequate to very specific applications such as for feeding small devices in remote locations. Normally, disposable batteries are used with this purpose, with the inconvenient that they must be replaced because of the limited service life. This is one of the scenarios that can take more advantage from miniaturized MFCs in the future (Ren *et al.*, 2012).

Comparing traditional bioenergy technologies, the MFC technology has the following advantages:

- Broad fuel availability. A wide number of organic matters such as wastewater, sludge and biomass can be utilized as fuel in MFC for electricity production.
- Clean production process and products. A MFC has no substantial intermediary processes and the energetic product, the electricity, is kind of energy ready to use.
- Low cost of catalyst instead of expensive metals.
- Broad applications. MFC can be utilized for wastewater treatment, pollutant removal, hydrogen production and electrosynthesis (Logan and Regan, 2006; Rabaey and Verstraete, 2005).

Nevertheless, the electricity that can be harvested from MFCs (is much lower than those in, for example, hydrogen fuel cells. Typical maximum power densities in MFCs are ~2 to 3 W m^{-2} of projected electrode (usually the cathode) and under optimum temperature of ~30°C, and well-buffered medium.

Microbial Electrosynthesis Cells (MES)

Microbial electrosynthesis is the probably the most emerging area in microbial electrochemical research. In these systems, the electrogens use the electrons derived from the cathode to reduce carbon dioxide and other chemicals into a variety of organic compounds. In general, acetogenic bacteria use hydrogen as the electron donor, and CO_2 as carbon source. However, it was found that a cathode could also serve as an electron source for producing organic acids for acetogenic species such as *Clostridium ljungdahlii*, *Clostridium aceticum*, *Sporomusa sphaeroides* and *Moorella thermoacetica* (Nevin *et al.*, 2011). Methanogens can also accept electrons directly from cathodes (Clauwaert *et al.*,

2008) to produce hydrogen, which can be further converted to methane in an external anaerobic digester.

MESs are mainly focused on synthetizing compounds with multiple carbons that can be precursors for desirable value-added chemicals or liquid transportation fuels (Marshall *et al.*, 2013; Rabaey and Rozendal, 2010). For instance, acetate (Jourdin *et al.*, 2016; Patil *et al.*, 2015) and butyrate (Ueki *et al.*, 2014) can be synthesized from carbon dioxide. It has also been shown that ethanol can be produced from acetate at the cathode, sometimes with the addition of mediators as methyl viologen (Steinbusch *et al.*, 2010). Additionally, other inorganic chemicals have been produced in the cathode chamber as struvite from phosphate recovery (Cusick *et al.*, 2014) ($MgNH_4PO_4 \cdot 6H_2O$) in a modified microbial electrolysis struvite-precipitation cell (MESC). Rozendal *et al.* showed that hydrogen peroxide can be produced by reducing oxygen at the cathode (two electron reduction process) (Rozendal *et al.*, 2009), coupled to the microbial oxidation of organic matter in the anode, at an applied voltage of 0.5 V (ΔE). Compared to conventional electrochemical methods, the H_2O_2 production in MECs requires much lower energy.

In general, microbial electrosynthesis provides a highly attractive and novel route that might convert solar energy to valuable products more effectively than traditional approaches.

Microbial Desalination Cells (MDCs)

The basic principle of MDCs is to utilize the potential gradient generated across the anode and cathode to drive desalination in situ. Compare to other METs, MDCs have a third chamber for desalination by inserting an anion exchange membrane (AEM) and a cation exchange membrane (CEM) in between the anode and cathode chambers (Figure 1-8). When electroactive bacteria in the anode chamber oxidize organic substrates and produce electrons and protons, the anions (*e.g.*, Cl^-) from the salty water in the middle chamber migrate to the anode and the cations (*e.g.*, Na^+) are drawn to the cathode for charge balance, thus the middle chamber solution is desalinated (Cao *et al.*, 2009; Luo *et al.*, 2012). MDC became of great interest because it can be used as a stand-alone technology for simultaneously removing organics and salt with energy production (Saeed *et al.*, 2015). Either, MDC can be utilized as a pretreatment for conventional desalination processes such as reverse osmosis to reduce the salt concentration in the influent, and minimize energy consumption and membrane fouling (ElMekawy *et al.*, 2014).

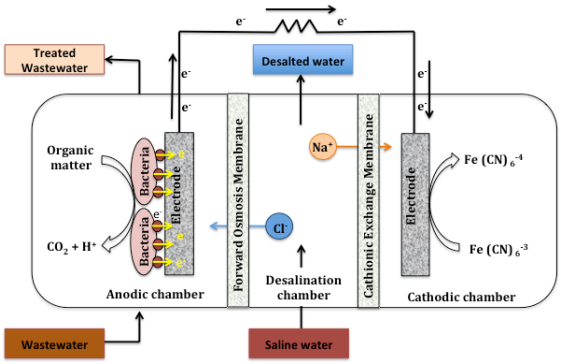

Figure 1-8: Schematic of a typical Microbial Desalination Cell (MDC) of 3 cameras, without external power supply. The current flow comes from the microbial oxidation at the anode of organic matter and the cathodic reduction reaction is Fe (III)/Fe(II).

Microbial Electroremediating Cells (MERCs)

Another emerging environmental application of METs is using the electrodes to serve as electron acceptors (anode) or donors (cathode) for removing contaminants from soils or sediments (Huang *et al.*, 2011; Morris and Jin, 2008; Yuan *et al.*, 2010). These MFC systems were firstly called Microbial Remediating Cells (MERCs) by Rodrigo *et al*, and described as devices to overcome electron acceptor limitation and maximize metabolic oxidation (Rodrigo *et al.*, 2014). Like sediment MFCs, MERCs used in groundwater or soil remediation can be a single or an array of electrodes directly located at the polluted area. This presence of a conductive surface can stimulate microbes to concurrently degrade underground pollutants and produce additional electricity. The supply of an electron acceptor (anode) for microorganisms eliminates the injection of expensive chemicals and reduces operational energy cost as compared to other technologies.

Zhang *et al.* (2010) reported for first time this new concept for bioremediation for the degradation of toluene and benzene in polluted slurries (Zhang *et al.*, 2010). Since then, several studies have reported the biodegradation enhancement of pollutants of different chemical nature: PAHs (Chandrasekhar and Venkata Mohan, 2012; Huang *et al.*, 2011a; Rodrigo *et al.*, 2014; Yan *et al.*, 2012b), herbicides as isopropuron (Rodrigo Quejigo *et al.*, 2016) or atrazine (Dominguez-Garay A., 2016); chlorinated compounds (Chun *et al.*, 2013; Liu *et al.*, 2013; Yu *et al.*, 2016) and pesticides (Cao *et al.*, 2015; Liang *et al.*, 2014). Importantly, MERCs do not also stimulate the degradation of pollutants, but also enhance its mineralization to CO_2 and decrease the toxicity of the soil (Rodrigo Quejigo *et al.*, 2016), proving to be an

effective bioremediation tool. Rodrigo *et al* named this effect as bioelectroventing due to the enhancement of bioremediation under soil-flooded conditions by using electrodes as microbial electron sink.

Biosensors

MECs can also be used as a new kind of biochemical sensor. The correlation between current generation and substrate concentration has been widely used as the basis for biological oxygen demand (BOD) sensors. In the wastewater field, researchers have successfully developed miniaturized biosensors based on MFCs for measuring the BOD (Peixoto *et al.*, 2011), acetate (Li *et al.*, 2011), pH (Uria *et al.*, 2016) as well as toxic compounds (Dávila *et al.*, 2011; Liu *et al.*, 2014a). A novel approach on that is the immobilization of cells inside silica gel and carbon felt as a new strategy for constructing ready-to-use artificial bioelectrodes of *G. sulfurreducens* (Estevez-Canales M., 2016). The synthetic biology is getting much attention because of the potential possibility of building *ad hoc* cells for biosensors with high robustness, sensitivity and specificity (Bereza-Malcolm *et al.*, 2015). For instance, it has been achieved by genetic engineering a modified *E.coli* (non-electrogenic bacteria) hosting a portion of the extracelular electron transfer chain of *Shewanella oneidensis* MR-1. However, the engineered *E.coli* was able to perform EET at very low rates (Jensen *et al.*, 2010).

In addition to the use of biosensors for measuring a chemical property, it has also been developed the concept of biosensors as a tool for assessing the microbial electroactivity by employing screen-printed electrodes, a novel low-cost platform at the microscale level (Estevez-Canales *et al.*, 2015). This was also used for characterizing the response of *G. sulfurreducens* under diverse physiological states revealing different electron transfer responses.

Metal Removal And Recovery

Bioelectrochemical technology can be used for the metals from the waste streams from the mining and metallurgical industry. Base metals like copper, nickel, iron, zinc, cobalt and lead, are used in large quantities and are ubiquitous in process. METs have already demonstrated to remove and recover metals from different wastewaters through the oxidation and reduction reaction oriented processes in either the anode or cathode. Four mechanisms have been reported so far (Wang and Ren, 2014): a) direct metal recovery using abiotic cathodes in MFCs (for those with redox potential higher than the anode potential like Au (III) (Choi and Hu, 2013), V (V) and Cr (VI) (Zhang *et al.*, 2012), Cu (II) (Heijne *et al.*, 2010) or Zn (II) (Fradler

et al., 2014b)); b) metal recovery using abiotic cathodes supplemented by external power sources (for metals with a lower redox potential like Ni (II) (Qin *et al.*, 2012), Pb (II), Cd (II) or Zn (II) (Modin *et al.*, 2012)); c) metal conversion using biocathodes (like Cr (VI) (Daulton *et al.*, 2007; Gangadharan and Nambi, 2015)); and d) metal conversion using biocathodes supplemented by external power sources (reduction of Cr (VI) at higher rates (Huang *et al.*, 2011b).

This process can be coupled to the degradation of a waste. For instance, it has been previously described that Cu^{2+} can be reduced to metallic copper on the cathode of a MFC coupled to the microbial oxidation of organic matter and the electricity generation (Heijne *et al.*, 2010; Rodenas Motos *et al.*, 2015). It has also been reported the uranium removal and recovery from contaminated sediments with poised electrodes serving as electron donors for microorganisms (Gregory and Lovley, 2005).

The main drawback of the microbial mediated process is that high concentration metal solutions generally inhibit microbial activities and reduce system efficacy. The abiotic process in the cathode usually employs low catholyte pH in order to keep metal dissolved in acidic condition, which can be problematic from the environmental and the operational point of view.

Wastewater Treatment

The successful application of METs in the wastewater treatment field is probably, regarding the large amount of studies and the environmental impact, the most relevant challenge that face these technologies. Thus, the next section has been entirely dedicated to describe the fundaments and the state of the art of the microbial electrochemical wastewater treatment.

1.3 METs As A Novel Technology For Wastewater Treatment

The requirements of new environmental legislation on municipal and industrial wastewater eflluents have driven researchers to find more efficient technologies for wastewater treatment that minimize both the energy demand and the final waste while reusing the by-products generated. In this regard, some research lines are focused on alternative forms of microbial metabolisms (*e.g.* anammox (Mulder *et al.*, 1995) and microbial electrochemistry (Lovley, 2006)) while others try to optimize the already existing configurations. The use of electroactive bacteria has been extensively reported as having a large potential for wastewater treatment (Borjas *et*

al., 2015; Du *et al.*, 2007; Modin and Gustavsson, 2014; Rozendal *et al.*, 2008). A wide range of organic compounds have been reported to be suitable as electron donors for microorganisms that use an anode as their terminal electron acceptor (Jung and Regan, 2007) (see Figure 1-9). Likewise, electroactive bacteria can also use a cathode as electron donor for reducing a variety of substrates such as nitrate (Clauwaert *et al.*, 2007), nitrite (Puig *et al.*, 2011a), sulphate (Coma *et al.*, 2013), CO_2 (Nevin *et al.*, 2010), tetrachloroethane (Strycharz *et al.*, 2008), etc.

The attractive aspects and the advantages of the microbial electrochemical wastewater treatment over other treatment methodologies are the following:

- Large variety of substrates can be oxidized or reduced bioelectrochemically.
- Less sludge production, due to the low cell growth of the anaerobic electroactive bacteria compared to the aerobic metabolism.
- Clean production process and products. The direct conversion of a substrate to electricity means energy ready to use. The off gas is CO_2, which can be discharged without further treatment.
- Mild operating conditions. Unlike anaerobic digestion and other fermentation processes, METs can be applied at low temperatures and can treat low strength wastewaters.
- The aeration step can be eliminated, and therefore the consequent associated costs.
- Compared to other electrochemical methods, METs use low cost catalysts, the microorganisms, instead of expensive metals.
- The potential for producing chemicals such as H_2 and hydrogen peroxide from wastewater treatment
- The use of electrochemistry allows to fine tune the microbial reactions at the anode and cathode by controlling the potential of this electrodes or the current flowing between them.

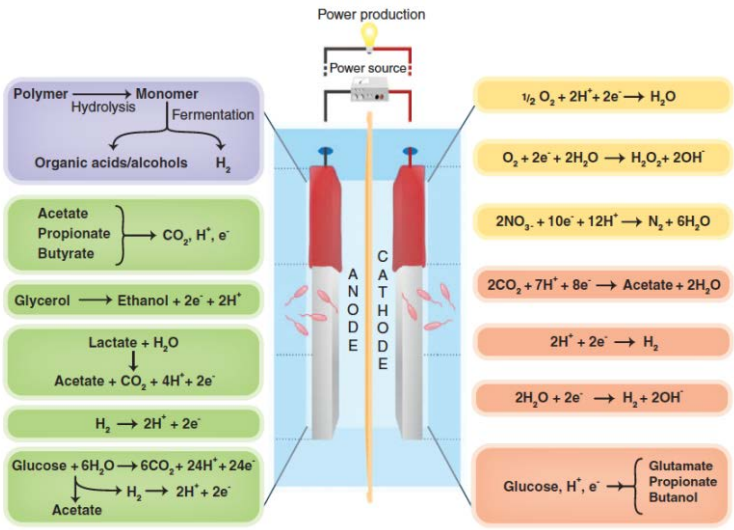

Figure 1-9: Overview of reactions that can be performed by electroactive microorganisms in the anode and in the cathode of a MET. The reactions in purple do not produce an electric current. The green ones can produce and electric current. The reactions in yellow can be spontaneous or accelerated by adding power. The reactions in orange require the addition of power. From Logan and Rabaey, 2012 (Logan and Rabaey, 2012).

1.3.1 Fundaments And Objectives

Bioelectrochemical Organic Mater Removal

Microbes in the anodic chamber of a MET utilize electrons and protons to accomplish organic substrate degradation under anaerobic conditions. Figure 1-9 shows the range of substrates that electroactive bacteria can directly utilize is broad: VFAs, ethanol, H_2. If more complex substrates are present in the wastewater, then the electrogenic metabolism needs of a partner that breaks these compounds into more simple molecules. For instance, it was reported cellulose degradation coupled to current generation in a MFC using a defined coculture of a fermenter and *G. sulfurreducens* (Ren *et al.*, 2007). The process was based on a first conversion of the cellulose to VFAs, and afterwards, the bioelectrochemical oxidation of the latter ones by the electroactive culture. When real wastewaters are treated, the electrochemical performance is importantly decreased compared to using synthetic water with easier biodegradable substrates. This is due to the low degradation rates of complex organic matter and to the appearance of competing processes as methanogenesis.

From the very beginning in the METs field, the bioelectrochemical treatment of domestic wastewaters has been the center of attention of researchers (Min and Logan, 2004; Rozendal *et al.*, 2008). The potential advantages derived from using an electrogenic metabolism instead of an aerobic one, which has traditionally been used in urban wastewater treatment plants (WWTPs), have been the main stimulating factor. However, currently a wide range of wastewaters have been successfully treated by METs: brewery effluents (Dong *et al.*, 2015; Wang *et al.*, 2008; Yu *et al.*, 2015), cheese industry wastewaters (Kelly and He, 2014a), palm oil mill effluents (Baranitharan *et al.*, 2015), wine lees (Cercado-Quezada *et al.*, 2010), yogurt waste (Cercado-Quezada *et al.*, 2010), swine wastewaters, (Lim *et al.*, 2012; Min *et al.*, 2005), rice mill effluents (Behera *et al.*, 2010), etc. Food industry effluents have gained much attention since their organic matters are easily oxidized by microorganisms and thus are considered as ideal fuels for METs. For instance, brewery wastewater treatment has been succesfully treated in a 90 L stackable baffled MFC with a net energy generation of 21-34 W m^{-3} and organic matter removal up to of 88% (Dong *et al.*, 2015). Zuang *et al* (2011) designed a 10-liter serpentine-type MFC for the treatment of brewery effluent as well, achiving power outputs of 4.1 W m^{-3} (0.7 A m^{-3}), COD removals of 0.9 kg COD d^{-1} m^{-3} and CE of 8% (Zhuang *et al.*, 2012a). They observed a decrease in electrical performance at long term due to a cathode limitation provoked by alcalinization over time.

Landfill leachate treatment has also been tested in MFCs, with electricity production (344 mW m^{-3}) coupled to the organic matter removal of 8.5 kg COD d^{-1} m^{-3}, and under high levels of ammonia (6 g-N L^{-1}) (Puig *et al.*, 2011b).

The performance of the treatment depends highly on the wastewater composition. It has been observed that at higher organic matter concentrations, increasing amounts of organic matter can be removed, but results in decreasing coulombic efficiencies. This is due to the stimulation of other microbial processes such as fermentation and methanogenesis (Freguia *et al.*, 2007; Logan and Regan, 2006).

The materials used for the anodes are commonly carbon based materials such as carbon and graphite due to their stability when microbial cultures are grown on them and to their relatively low cost. Stainless steel has also been widely used, but the active surface area of this material is much lower as compared to the ones achieved with carbon materials such as felt, granules, or fibers. Larger surface areas provide more space for microbial attachment, which results in higher electron transfer and reaction rates per volume of treated wastewater.

Bioelectrochemical Nutrients Removal & Recovery

a) Nitrogen

In conventional wastewater treatment systems, the organics available in the wastewater are typically used as electron donor during denitrification. In METs, a cathode can serve as electron source for heterotrophic or autothrophic electroactive microorganisms to reduce the nitrate (Figure 1-10). The electrons provided by a biocathode can come either from acetate oxidation in a bio-anode or from the abiotic electrochemical oxidation of other compounds such as water. Since the electron flux through the METs electrodes can be tuned, fine control over the rate of denitrification can be performed in these systems so that treatment requirements can be achieved. All of these features make a microbial electrochemical system a potential alternative for removing nitrogen from wastewaters with low organic matter content, or even from groundwater (Pous *et al.*, 2013; Tong and He, 2013).

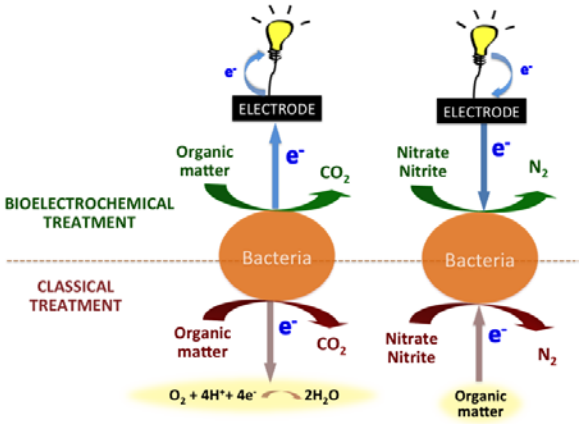

Figure 1-10: Bioelectrochemical nitrogen and organic matter removal process in wastewater.

The first study that reported the bioelectrochemical reduction of nitrate was in 2004 (Gregory *et al.*, 2004), with *Geobacter metallireducens* using a graphite electrode as sole electron donor to convert nitrate to nitrite, the fist out of four steps for denitrification to dinitrogen gas (ec. [3], [4], [5], [6])[2]. The bioelectrochemical denitrification process has been achieved with pure cultures and with mixed populations (Arredondo *et al.*, 2015; Puig *et al.*, 2011a; Sayess *et al.*, 2013; Tong and He, 2013; Yan *et al.*, 2012a).

[2] *The standard electrode potential of Ag/AgCl (in saturated KCl) against SHE is +0.2 V at 25 ºC.*

$NO_3^- + 2e^- + H^+ \rightarrow NO_2^- + H_2O$ E^o=+433 vs SHE [3]

$NO_2^- + e^- + H^+ \rightarrow NO\ (g) + H_2O$ E^o=+350 vs SHE [4]

$NO\ (g) + e^- + H^+ \rightarrow ½\ N_2O\ (g) + ½\ H_2O$ E^o=+1.175 vs SHE [5]

$½\ N_2O\ (g) + e^- + H^+ \rightarrow ½\ N_2\ (g) + ½\ H_2O$ E^o=+1.355 vs SHE [6]

Regarding the reactor configurations, different designs have been proposed for performing a complete nitrogen removal treatment. For instance, nitrification can be accomplished in a separate chamber, transforming the ammonium to nitrite or nitrate, while denitrification can be carried out in the cathode, as previously described by Virdis *et al.* (Virdis *et al.*, 2008). Other studies have investigated the simultaneous aerobic nitrification/bioelectrochemical denitrification in the same chamber (Sayess *et al.*, 2013; Virdis *et al.*, 2010).

METs also offer an opportunity for direct recovery of ammonium nitrogen in the form of NH_3. The basis of this phenomenon is that when current is generated in an electrochemical cell, the ammonium ions get transported from the anode chamber to the cathode chamber chiefly by two distinct processes such as diffusion and migration. Ionic ammonium is then transformed into volatile ammonia at the cathode (due to the pH increase), which can be removed from the cathode compartment by NH_3 stripping with a suitable gas stream. The current driven migration of ammonium ions can be of paramount importance for developing recovery systems coupled to wastewater treatment. Urine (Kuntke *et al.*, 2012) and swine wastewater (Kim *et al.*, 2008) are a potential resource for recovering ammonium nitrogen for reuse in this technologies. It has also been investigated the simultaneous ammonium recovery and hydrogen production from urine in an MEC, with a maximum nitrogen removal of 173.3 g-N m^{-2} d^{-1} at a current density of 23.1 A m^{-2} (Kuntke *et al.*, 2014). For each kg of nitrogen recoverd in a MET, 0.57 kg of COD is required (assuming that 1 mol of COD corresponds to 4 mol of electrons, a 100% of CE, that all cation transport occurs through NH_4^+, and that all NH_4^+ is recovered at the cathode) (Arredondo *et al.*, 2015). In contrast, for nitrification/denitrification, theoretically, for removing 1 kg of nitrogen, 2.86 kg COD is required, while nitrification and Anammox require no COD at all.

b) Phosphorous

Phosphorous is usually removed via chemical precipitation (Tchobanoglous and Burton, 1991) (through the addition of calcium, aluminium or iron), physical treatments (González *et al.*, 2002) (as reverse osmosis), physico-chemical methods (as electrocoagulation) (İrdemez *et al.*, 2006) or biological processes (Kerrn-

Jespersen and Henze, 1993) (through aerobic-anaerobic sequential steps) in WWTPs.

Phosphorous removal has not been such studied as nitrogen removal in METs. However, there is certainly an interest due to the importance of this pollutant in wastewaters and as valuable product. The main advances in BESs in this regard have been conducted towards the recovery of this pollutant as struvite mineral ($MgNH_4 \cdot PO_4 \cdot 6H_2O$). Several studies have investigated the precipitation of phosphorous as struvite at the cathode of MECs (Cusick and Logan, 2012; Cusick *et al.*, 2014; Fischer *et al.*, 2011) and of a MFC (Ichihashi and Hirooka, 2012), and all of them coupled the process to the oxidation of organic matter from a wastewater at a bioanode. This precipitation of phosphorous compounds is caused by the local pH increase at the vicinity of the cathode that results from the reduction reactions. The efficiency of this process depends highly on the cathode active surface, the pH and the voltage applied. The main challenges that these METs need to address are related to the long-term operation. Aspects such as the influence of the struvite precipitates on current generation or the cathode design that allows the collection of precipitates without stopping the operation of the reactor, are some of the main points that need to be deeply studied on future investigations (Kelly and He, 2014b).

1.3.2 Scaling Up METs In WWTP: State Of The Art

Current Bottlenecks And Challenges

Lower performance and efficiencies have been achieved with larger reactors than the tested at laboratory scale (Dewan *et al.*, 2008). The main limiting factors are related to the costs of the materials, the treatment capacity, and the energy demand of the potentiostat or power source if used. The latter one is a function of a large number of factors, from the pure electrochemical ones, to the biological aspects of the technology. The main bottlenecks in the scaling-up process of METs in wastewater treatment plants are briefly described in the following lines and have been divided as a function of the MET element which affects to (Logan and Rabaey, 2012).

Electrodes

Cost of materials: some materials used in laboratory-scale setups, such as carbon cloth and platinum electrodes, are too expensive for full-scale wastewater application that will need large electrode surfaces. Instead, electrodes made of materials that present high active surface area like graphite or carbon granules, and

that present a relative low cost, are emerging for microbial electrochemical wastewater treatment (Escapa *et al.*, 2016). Surface modification (with ammonia treatment, acid or heat treatment, etc) has provided with good results on the bacteria adhesion and electron transfer (Kumar *et al.*, 2013). The materials used in METs are typically carbon-based, which, compared to metallic-like electrodes, are less electrically conductive. This term of resistance cannot be relevant at laboratory-scale but as electrodes are scaled-up, the term becomes important.

- **Biofouling:** solids and biomass can lead to clogging and the active surface area of the electrodes can be severally reduced.

Biofilm electroactivity

- **Competition** of bacteria for space on the electrode: non-electrogenic bacteria attach to the electrode could occupy electrode space that could instead be used by electrochemically active bacteria. This causes non-desired reactions and a discontinuity on the possible conductive network of the biofilm. Inert material, as solids, can also limit the electrode active surface for electrogens.

- **Degradation and electron transfer rates**: one of main goals of the field is to be able to control the activity and growth of the electroactive microorganisms in order to guarantee a stable performance of the system (Pham *et al.*, 2006). Thus, the study and optimization of the microbial kinetics and the mechanisms involved in the exocellular electron pathway of cells and biofilms could play a relevant role on the acceleration of the catalytic process.

- **pH gradients:** the production of protons at the vicinity of the anode and OH^- ions at the cathode can affect the viability of the electroactive biofilm if the diffusion within the biofilm is slow (Torres *et al.*, 2008). In addition, it also can lead to a loss of electrochemical potential.

- **Sustained biocatalyst activity over time:** the microbial activity in METs is still not well understood. The structure and evolution of the microbiome in an electrode and the role of the members of each community in the catalysis is fascinating and very complex unknown information.

- **Coulombic efficiencies:** the term of coulombic efficiency (CE) refers to the proportion of electrons utilized for catalyzing bioelectrochemical reactions, resulting in a current flow, over the total amount of electrons obtained from substrate oxidation. Using well-buffered systems, simple compounds as acetate, formate or lactate, and pure electroactive cultures, the CE can reach values up to 93 % (Speers and Reguera, 2012). However, the CE values reported for different real wastewaters

have been typically in the range of 5-10 % (Pant *et al.*, 2010). This is due to the competitive parallel reactions that occur in the biofilm or by planktonic biomass in the wastewater when working with mix populations and complex substrates. One of the goals in METs field is to be able to control these alternative metabolisms by maintaining certain environmental conditions at which electrogens outcompete the rest of the microbial community.

Wastewater

• **Conductivity:** the conductivity of real wastewaters is low compared to the synthetic media with high ionic content that are used at laboratory scale. This is one of the constraints of treating wastewater with electrochemical methods. The ohmic losses in these systems, related to the ion migration, are usually high and therefore the anode and cathode distance must be small to minimize this loss.

• **Development of *ad hoc* bioelectrochemical treatments**: not all the METs are appropriate for treating all kinds of wastewater. The most effective systems would be those ones tailored for each influent and effluent quality needs. In addition, METs cannot completely perform the treatment of a wastewater by themselves and it becomes necessary the complementation of them with other technologies. For example, a previous anaerobic digestion step could reduce the COD of the influent of a MET and thus enhance the performance of the bioelectrochemical reactor.

Reactor design

Figure 1-11 shows some examples of configurations that have been used from the very beginning in the laboratory (as the H-shaped cell) until nowadays (as the filter press-based bioelectrochemical design). For the scaling-up process, continuous flow (lower residence time values and large water volumes treatment), single-compartment METs and membrane-less are favored for wastewater treatment. Wastewater infrastructure is expensive to build and typically is designed to last at about 50 years. One of the challenges on the reactor design is be able to exploit the existing built structures of wastewater treatment plants. This would eliminate a great part of the initial investments costs associated to the implementation of a bioelectrochemical wastewater treatment.

Configurations And Designs Utilized

METs have been mainly based on devices in which the biocatalysis is located at the electrode interface due to the need of microbial attachment. The process for implementing these technologies to full scale requires the study of new scenarios that overcome the limitations of the biofilm-based systems. Some of these

constraints could be reduced using a configuration where every single cell in the culturing medium could be wired to the electrode. Mediated electron transfer might aid in this regard. However, while this method can enhance the output of current, it also suffers from two main drawbacks: first, the production of endogenous mediators is highly energy-demanding for the cells; and secondly, a continuous mode of operation would negatively influence the concentration of the electron shuttle in the reactor (Schröder, 2007).

For maximizing the connections between microbes and electrodes, most of the efforts have been addressed towards increasing the active area of the catalysis. In this sense, three-dimensional bed METs can increase the surface area of the anode for bacteria adhesion. The bed can remain fixed or in motion. Fixed bed-METs provide a large ratio of electrode area per volume of wastewater. Regarding the materials used, granular activated carbon has been implemented as the packing material in MFC to treat domestic wastewater (Jiang and Li, 2009). More recently, biochar-based systems have shown excellent treatment performances as both anode and cathode in MFCs (Huggins *et al.*, 2014, 2015).

METlands (wetland plus a MET) are also fixed-bed designs that have resulted from merging constructed wetlands for wastewater treatment with METs (Figure 1.11.F). They have show to enhance the biodegradation rates in wastewater treatment or to reduce the classical constructed wetland dimensions (Aguirre-Sierra A. *et al*, 2014). The group of Bioelectroegenesis of Dr. Abraham Esteve-Núñez developed a full-scale METland in Carrión de los Céspedes, Spain, which treated cubic meters of real wastewater for over 2 years (Esteve-Núñez A., 2014). This technology was able to attenuate the phenomena of clogging of the filter substrate. The project iMETland (European Union's Horizon 2020 research and innovation program), currently ongoing, aims to implement this technology in small communities at zero-energy cost and with remote control process.

In contrast, dynamic beds as in fluidized reactors, or in up-flow anaerobic sludge beds can be converted into electrodes serving to microbes as both carriers and electron acceptors or donors. In addition, novel scenarios have been proposed for removing the organic matter in wastewaters, like the systems with carbon-based capacitive mobile granules. These granules are covered by an electroactive biofilm that transfer the electrons resulting from its metabolism to the conductive material. Afterwards, the charged granules are recirculated to the anodic chamber of an external MFC and transfer the electrons to a current collector (Deeke *et al.*, 2015), remaining oxidized and acting as an electron sink again. These kinds of mobile 3D-

electrode configurations present good mass transport properties, mixing, and temperature distribution, besides a large active surface area.

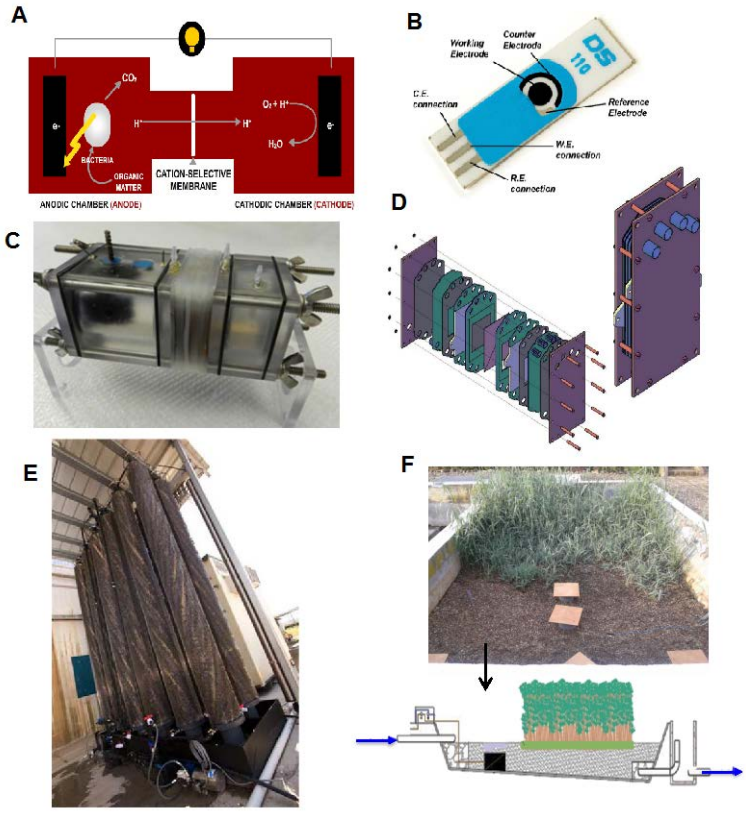

Figure 1-11: Pictures or schemes of different METs configurations. A.Lab-scale H-shaped cell. B: Carbon screen-printed electrode for micro-scale and quick assays (from Dropsens, Llanera, Spain). C: Lab-scale 2 chamber microbial reverse electrodialysis cell with a carbon fiber brush as anode (Cusick *et al.*, 2012). D: Pre-pilot filter press-based bioelectrochemical reactor from Borjas *et at*, 2016. E: Tubular MFC system for brewery wastewater treatment (University of Queensland, AU). F: METland operating at Carrión de los Céspedes wastewater treatment plant (CENTA Foundation, Spain).

The development of hybrid MET-based systems, as METlands or membrane bioreactor MFCs (Malaeb *et al.*, 2013), is currently gaining much attention in the field (Xu *et al.*, 2016). Merging several technologies allows incorporating the respective merits of each individual technology into the same treatment.

1.3.3 Future Perspectives For The Bioelectrochemical Wastewater Treatment

Microbial Electrochemical Technologies have emerged as novel systems that fill well within the recently recognized water-energy nexus. Although there is a chance of generating electricity from the microbial oxidation of waste, the values are low and cannot compete with renewable energies as wind or solar. In addition, currently the value of electricity produced in a MFC is less valuable than the value of hydrogen. Therefore, the production of electricity will not necessarily be the main goal of METs in the future.

The real opportunity of METs in the wastewater treatment field relies on the energy-saving benefits and on the possibility of producing added value products as H_2, caustic soda (Rabaey *et al.*, 2010) or hydrogen peroxide from the wastewater treatment (Sleutels *et al.*, 2012). Although a small amount of energy is required for driving the production of these compounds in a MEC, the energy contained in them or the revenue offsets the energy initially needed to activate the process.

Proofs of concepts at larger scale need to be performed in order to test the reliability of these new technologies and its behavior at long-term operation. Thereafter, new problems and challenges related to real-world conditions, and different form those ones faced at the laboratory scale, will arise.

From The Laboratory To The Market And Society

Since the discovery of the diverse applications of METs on the wastewater treatment field, the interest on a transition towards the market is exponentially increasing. So thus, several spin-offs and R&D start-up companies that were born promoted by the scientific community are looking into commercialization of devices for the microbial electrochemistry field or directly METs for mostly treating water (Rosenbaum and Franks, 2013). For instance, Nanoelectra (www.nanoelectra.com) (Madrid), is a biotechnology start-up company that designs and develops hardware for the harvesting and utilization of the electricity produced by electrogenic microorganism, with a strong emphasis on microbial electrochemical applications for water and soil remediation. Another example is METfilter (Madrid) (www.metfilter.com), which constructs electrically conductive biofilters (METlands) for treating wastewater at full-scale. Emefcy Ltd. (Israel) (www.emefcy.com) and Cambrian Innovation (www.cambrianinnovation.com) (Boston) develop, among other technologies, microbial electrochemical systems for wastewater treatment. Currently, the idea of converting renewable electricity produced from wastwewater into storable chemical products is gaining much attention and boosting METs industrial

applications. In this regard, Electroarchaea (US-based start-up) (www.electroarchaea.com), and Bilexys (Australia) (www.bilexys.com), commercialize METs for producing chemicals like methanol, sodium hydroxide and hydrogen peroxide, at either the anode or the cathode of these devices.

Although there is still a lot of research required, the microbial electrochemistry field is finding niches for competing with current technology. This novel and fascinating field is inspiring new concepts for biotechnology providing increasing applications for the scientific community and the industry.

Objectives and Thesis Outline

The present thesis aims to evaluate novel scenarios based on merging microbial electrochemical systems with conventional reactors designs for treating wastewater. The challenge of these hybrid configurations is to overcome some of the technological, economical and microbiological bottlenecks for up-scaling METs. So thus, the following specific objectives were proposed:

- Objective 1: To design a MET that incorporates the fluidization concept used in traditional anaerobic reactors in order to create an environment that maximizes the electrode-bacteria interaction
- Objective 2: To study the capacity of fluid-like anodes to accept electrons from electroactive microorganisms. Also, to investigate both the features and the viability of this interaction.
- Objective 3: To operate and evaluate a microbial electrochemical fluidized bed reactor for treating a real industrial wastewater, providing insights into the microbial reactions of this electrogenic treatment
- Objective 4: To develop and integrate complementary technologies to the microbial electrochemical fluidized bed reactor for achieving a complete treatment of a real wastewater.

The research developed to achieve the objectives has been reported throughout the different chapters, except for Chapter 1 (introductory) and Chapter 5, (a general discussion, conclusions and future outlook). The remaining chapters correspond to articles published or submitted to peer-review journals.

Objectives 1 and 2 were treated in Chapter 2: *The planktonic relationship between fluid-like electrodes and bacteria: wiring in motion* and in Chapter 3: *Fluidized bioanodes vs non-conductive classical fluidized beds on the treatment of a brewery effluent.* In those chapters we present the concept of the ME-FBR and explore the bacteria-electrode interaction. In Chapter 2 we study the fundaments of that interaction by using the model bacteria *Geobacter sulfurreducens* in combination with acetate as substrate, and glassy carbon particles as anodic material.. In Chapter 3, we use a mix population in a ME-FBR for treating a real brewery wastewater at continuous mode. In this chapter we also deal with Objective 3, characterizing the treatment of the wastewater in the ME-FBR and comparing the electrogenic treatment with the one achieved in a standard fluidized bed reactor with non-conductive particles.

The Objective 4 is addressed in Chapter 5, which is divided in two parts. In Part 1: *Integrating a Microbial Electrochemical System into a classical wastewater treatment configuration for removing nitrogen from low COD effluents***,** we study the elimination of nitrogen in a post-treatment of low COD effluents (*e.g.* effluent from a ME-FBR) in a bioelectrochemical system. In Part 2: *Merging microbial electrochemical systems with electrocoagulation pretreatment for achieving a complete treatment of brewery wastewater*, we study the possibility of integrating an electrocoagulation pre-treatment to remove both nutrients and suspended matter.

Research Framework

The present PhD thesis was developed within the frame of three research projects, completely or partially focused on applying microbial electrochemical systems for treating wastewaters. The first one, Aqualectra (Bioelectrogenic treatments applied to wastewater treatment), was a project funded through the program INNPACTO from the Spanish Ministry of Science and Innovation. It was formed by a consortium of two research institutions: the Bioelectrogenesis group of IMDEA Water (Alcalá de Henares, Spain) and CENTA (Foundation Centre of New Water Technologies, Seville, Spain). The consortium was also formed by the water enginering companies DAM (Mediterranean Water Purification), JOCA and Euroestudios. This initiative aimed to provide the application of new techniques that allow, in addition to wastewater treatment, to obtain and store clean energy. The project aimed three objectives: a) to develop a natural system of sewage treatment using MET-based wetlands, b) to establish a MET-based anaerobic system for treating wastewater and c) to build a MET-based nutrient (nitrogen) removal system. The results presented in this thesis (Chapter 4, Part I) were related to the objective 4 aimed in Aquaelectra. On top of that, Aquaelectra project generated two patents corresponding to experiments not included in this thesis report:

- Patent 1: Authors: Esteve-Núñez, A., Tejedor-Sanz, S., Berná A., Salas J.J., Pidre J.R., Aragón C and López F. Title: *Bioelectroquímico system for treating wastewater with floating spheres conductive cathode*. Publication N.: ES2539510. Application N.: P201331937 Date of priority: 30/12/2013. Date of publication: 1/7/2015. Applicants: CENTA Foundation, IMDEA Water and JOCA.

- Patent 2: Authors: Esteve-Núñez, A., Tejedor-Sanz, S., Berná A., Salas J.J., Pidre J.R., Aragón C and Pastor L. Title: *Procedure for microbial nitrate removal in wastewater and electrogenic biological system*. Authors: Publication N.: ES2539416. Application N.: P201331936 Date of priority: 30/12/2013. Date of publication: 30/6/2015. Applicants: CENTA Foundation, IMDEA Water and DAM (Mediterranean Water Purification).

The second project, called Microbial Electrochemical Fluidized Bed Reactors: A New Concept Applied To The Degradation Of Pollutants In Water (2012), was funded by Alcalá University and Comunidad de Madrid (Program for Promoting the Creation and Consolidation of Research Groups). Within this project, the concept of merging a classical fluidized bed reactor and a MET was firstly explored as a new scenario in microbial electrochemistry.

Last, the autor participated as well in the Project ITACA (Research on treatment technologies, reutilization and control for the future sustainability of water treatment processes) from the INNPRONTA program funded by the Industrial Technological Development Centre (CDTI), and co-funded by FEDER Funds through the I+D+I Operative Program for the companies benefit (Technological Fund). 9 companies (FCC Aqualia, DAM, ADASA, DEISA, JAP, DOW Chemical, CESPA, HidroQuimica and Técnicas Reunidas) and 11 technological centers (including the Bioelectrogenesis group from Alcalá University) formed this consortium. The global objective of ITACA project was to investigate new concepts of urban and industrial wastewater treatment technologies that allows, in an efficient and sustainable way, to convert the current treatment process in a strategy for the reutilization of the waste and subproducts, and its energetic valorization, thereby minimizing the environmental impact. The results presented in this thesis (Chapter 2, 3 and 4, Part II) correspond to the part of research on new biological water treatments of the ITACA project, and were developed in the research institution Universidad de Alcalá and with the company FCC Aqualia. In addition, we collaborated with the company Mahou-San Miguel for developing a MET for treating the effluent from the brewering process as an alternative to an anaerobic digester step. One from the results obtained throughout ITACA project has been requested:

- European Patent applied: Authors: Esteve-Núñez A., Tejedor-Sanz S., Berná A., Rodrigo J., León P. Title: *Method for treating wastewater in a fluidized bed bioreactor.* Publication. N.: EP2927196A1. Applicants: Universidad de Alcalá, FCC Aqualia. App number: 14382131.2, 7/10/2015.

The research line followed in ITACA will be continued through the project Advanced Nutrient Solutions With Electrochemical Recovery (ANSWER), from European program LIFE Environment and Resource Efficiency (2016-2019). The purpose of this project was to demonstrate the technical and economic feasibility of electrocoagulation and MET-based treatments in small industry wastewater treatment plants (brewery or other food technology sector) for zero effluent discharge.

In 2016, the author was a visiting scientist for 2 months in the group of Dr. Cesar Torres at the Swette Center for Environmental Biotechnology at the Biodesign Institute at Arizona State University (Arizona, U.S.A.). During this stay, the author collaborated in studies focused on the fundaments of the electron transfer of electroactive bacteria. The author also gained insights into molecular biology

techniques such as FISH (Fluorescence In Situ Hybridization), and the results of the application of this method are shown in Chapter 3.

CHAPTER 2: The Planktonic Relationship Between Fluid-like Electrodes and Bacteria: Wiring in Motion

This section has been redrafted after:

Sara Tejedor-Sanz[a,b], Jose Rodrigo[a], Antonio Berná[c] and Abraham Esteve-Núñez[a,b]. 2016. *The planktonic relationship between fluid-like electrodes and bacteria: wiring in motion*. Submitted.

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The Planktonic Relationship Between Fluid-Like Electrodes And Bacteria: Wiring In Motion

2.1 Abstract

The capacity of some microorganisms to exchange electrons with electrical conductive materials as part of their metabolism is one of the most fascinating mechanisms in the current field of microbiology (Lovley, 2006). This phenomenon typically occurs in an electroactive biofilm where all bacterial layers contribute to convert oxidative metabolism into electrical current (Erable *et al.*, 2010; Gimkiewicz and Harnisch, 2013; Schrott *et al.*, 2011). The main drawback of this scenario is the limited number of reactive cells, which depends upon the active area of the electrode wherein the bioelectrochemical reaction is performed (Jana *et al.*, 2014). We show, for the first time, that living in a biofilm is not a strict requirement for *Geobacter sulfurreducens* to exchange electrons with an electrode. We have explored a new concept in bacteria-electrode interaction based on the use of fluid-like electrodes and planktonic living cells. The growth of planktonic electroactive *G. sulfurreducens* could be supported by a fluid-like anode as soluble electron acceptors do and with electron transfer rates similar to those reported for electroactive biofilms. This growth was maintained by uncoupling the charge (catabolism) and discharge (extracellular respiration) processes of the living cells. Interestingly, the planktonic cells grown respiring a fluid-like anode showed an initial rate of iron-oxide reduction 10-fold higher than fumarate-grown cells did. This means that the fluid-like anode respiration stimulated the expression of similar strategies with those responsible for the electron transfer to iron oxides. Our results revealed a novel mode to culture electroactive bacteria where every single cell in the medium could be instantaneously wired to a fluid-like electrode. This culturing mode displayed a phenotype with a rapid metal-reduction capacity. Direct extracellular electron transfer is occuring but with a new paradigm behind the bacteria-electrode interaction.

2.2 Introduction

Since the discovery of *Geobacter* species almost 30 years ago, they have been presented as fascinating microorganisms due to their ability to perform extracellular electron respiration on Fe-oxides, uranium, vanadium, humic acids and, more recently, electrically conductive materials (Lovley *et al.*, 2011). Such a capacity

for exchanging electrons with electrodes have allowed the research community to investigate a number of microbial electrochemical systems having a plethora of applications in wastewater treatment, soil bioremediation, biosensors, microbial desalination or bioelectrosynthesis to name a few (Rosenbaum and Franks, 2013). So far, all of these technologies share a common scenario: the growth of an electroactive biofilm on the electrode surface. The biofilm-electrode interface has been investigated in depth by merging the most advanced electro-spectroelectrochemical techniques with *in vivo* assays. In this regard, *c*-type cytochromes were identified by Surface Enhanced Infrared Reflection Absorption Spectroscopy (SEIRAS) and Raman Spectroscopy as ultimately responsible for conducting the electrons from the outermost membrane of *Geobacter sulfurreducens* to the surface of a gold electrode (Busalmen *et al.*, 2008, 2010). Most efforts to optimize extracellular electron transfer (EET) to electrodes has been directed towards exploring biofilm-based systems (Erable *et al.*, 2010; Gimkiewicz and Harnisch, 2013; Katuri *et al.*, 2010; Schrott *et al.*, 2011). In principle, this seems reasonable due to the requirement of redox chemistry existing between cells and an insoluble material that would be satisfied by a biofilm architecture. Unfortunately, the main drawback of this scenario is the limited number of reactive cells, which depends upon the active area of the electrode wherein the bioelectrochemical reaction (first 50 µm-layer of the biofilm) is occurring (Jana *et al.*, 2014; Virdis *et al.*, 2014). In contrast with the electroactivity shown in a *Geobacter* biofilm, this bacterial genus is actually planktonic in their natural habitat, groundwater (Díaz, 2008). Furthermore, they are more physiologically active under this freely suspended life-style when growing with insoluble iron oxides as the sole electron acceptor (Childers *et al.*, 2002; Holmes *et al.*, 2002). Using this natural process as the driving force for our research, we hypothesized that a new electrode design could be able to simulate the dispersed insoluble iron state. This could provide more efficient electron transfer with practical applications. There is already some evidence suggesting that *G. sulfurreducens* planktonic cells are able to be electroactive (Esteve-Núñez *et al.*, 2011) after storing electrons in their *c*-type cytochrome network (Esteve-Núñez *et al.*, 2008). This was demonstrated when chemostat-grown cells were able to generate a rapid electrical discharge as soon as they were exposed to an electrode. These cells were so-called *plug-and-play* cells because their electroactivity allowed a major reduction in the start-up period of microbial electrochemical bioreactors (Borjas *et al.*, 2015; Esteve-Núñez *et al.*, 2011). In this work we have explored the interation of this *plug-and-play* cells with a fluidized anode, and have studied the ability of *G. sulfurreducens* to grow under that scenario.

2.3 Materials And Methods

Bacteria Strain and Growth Conditions. Cells of *Geobacter sulfurreducens* (strain DSM 12127; ATCC 51573) were grown at a fixed growth rate of 0.04 h^{-1} under continuous culture in a 2 L chemostat, at 30 ºC. The minimal medium composition (freshwater medium) was limited in electron acceptor (fumarate 10 mM) with acetate in excess (10 mM) as elsewhere reported (Esteve-Núñez *et al.*, 2005). In addition, *G. sulfurreducens* was also grown under batch conditions with acetate (20 mM) and fumarate (40 mM), using serum bottles sealed with butyl septum under an anoxic atmosphere of N_2:CO_2 (80:20) at 30 ºC. *G. sulfurreducens* cells with low *c*-type cytochromes content were obtained by culturing the strain under batch conditions but in presence of the iron chelator 2,2'-bipyridine as previously reported (Estevez-Canales *et al.*, 2014).

The Microbial Electrochemical Fluidized Bed Reactor. The ME-FBR consisted of a glass column (4.6 cm of internal diameter (ID) and 30 cm height) with a conical-shaped bottom (4.6 cm in ID and 5.2 cm height). The top of the ME-FBR was sealed during all the experiments in order to maintain an anoxic environment. For fluidizing, a recirculation flow was drawn from the top section using a peristaltic pump (Heidolph 5006). The medium was fed downwards by means of an elbow that drove the flow to the vertex of the conical bottom, resulting in a rising flow of fluid through the column. The total working volume of the reactor was of 0.63 L (including the recirculation tube and the bed volume). A graphite plate (20x80 mm) was used as a current collector and was was vertically inmersed in the fluidized bed. A platinum wire (0.1 mm thickness, 200 mm of lenght) was also used as current collector in the assays for promoting the cell growth. The cathode consisted of a RGV 2000 graphite felt piece (10x7x0.6 cm) from Mersen. An Ag/AgCl 3 M NaCl electrode (BASI) was employed as a reference electrode. Supplementary Figure 2-1 shows the schematic of the system. Two different carbonaceous materials were used as bed particles in order to test the ability of these particles to form an interaction between cells and the fluid anode. For the discharging assays with *Geobacter* cells at different oxidation states and cytochrome content, the bed was composed of 67 g (80 mL) of graphite particles from 0.42 to 0.69 mm mesh, which was previously washed by 1 M HCl followed by 1 M NaOH. For the remaining experiments, we used beds composed of glassy carbon particles of 0.63 to 1mm in size (Sigradur G, HTW, Germany). The culture medium used was a minimal basal medium containing 100 mM of $NaHCO_3$, 0.5 g L^{-1} of NH_4Cl, 0.6 g L^{-1} $NaH_2PO_4 \cdot 6H_2O$, 0.1 g L^{-1} KCl, 10 mL L^{-1} of a mixed vitamin solution and 10 mL/L of a mixed mineral solution, as previously described (Lovley and Phillips, 1988). The

system was kept anoxic by gassing with a mixture of N_2:CO_2 (80:20) and buffered at pH 7 using bicarbonate.

Electrochemical Measurements. The ME-FBR was operated as a three-electrode electrochemical cell and the conductive bed worked as the anode by polarizing the bed at 0.4 V (all potentials are reported versus Ag/AgCl electrode). Two different potentiostats were used depending on the current requirements and the electrochemical analysis performed (NEV3 Nanoelectra, current range above 100 mA, and a μAutolab type II, current range below 100 mA). Cyclic voltammograms were performed at a scan rate of 0.005 V s^{-1}.

Analytical Methods. Acetate concentration was determined by using a HP series 1100 high-pressure liquid chromatograph coupled with a UV detector (210 nm), equipped with a Supelco C-610H column and using 0.1 % H_3PO_4 as mobile phase with a flow rate of 0.5 mL min^{-1}. Fe (II) produced from ferrihydrate reduction was measured using the ferrozine method as previously described (Stookey, 1970). Samples were taken with anoxic syringes and were immediately acidified by tenfold dilution in 2 M HCl. All the samples were incubated overnight at 4ºC and in dark, and were measured photometrically at 562 nm.

Synthesis of Ferrihydrite. The ferrihydrite was synthesized as previously described (Lovley and Phillips, 1986) and the product was washed five times with a tenfold volume of distilled water. The resulting ferrihydrite suspension was deoxygenated by flushing it with N_2 with continuous stirring, flushing the headspace and sealing the flask. The anoxic suspension was autoclaved.

Microscopic Analysis of Bacteria. Scanning electron microscopy (SEM) and fluorescence microscopy was used to study the bacterial colonization of the glassy carbon particles and the planktonic growth in the ME-FBR. All the samples collected were gently rinsed with sterile deionized water prior to its preparation. Samples for SEM were fixed with 5% (v/v) glutaraldehyde in cacodylate buffer (0.2 M, pH 7.2) and dehydrated through a graded series of ethanol solutions (25, 50, 70, 90 and 100 %; 10 min each stage). Subsequently, the samples were rinsed two times in acetone for 10 min and immersed in anhydrous acetone at 4 ºC overnight. Finally, the samples were dried in CO_2 at the critical point and coated with gold. Micrographs were taken using a scanning electron microscope DSM-950 (Zeiss). For fluorescence microscopy, the samples were incubated in the dark at room temperature for 15 min with SYTO9 stain (2 μL mL^{-1} of a 3.34 mM stock) and propidium iodide stain (2 μL mL^{-1} of a 20 mM stock) from a LIVE/DEAD BacLight bacterial viability kit. Aftewards, the glassy carbon particles were gently washed with

30 mM phosphate buffer and the cell suspensions were harvested (10.000 rpm, 8 min) and washed twice in buffer. Samples were analyzed with an inverted Eclipse Ti-U microscope. We also used transmision electron microscopy (TEM) to examine the planktonic cells grown in the ME-FBRs. Samples were resuspended in cacodylate buffer (0.2 M, pH 7.2) and were negatively stained with 2% uranyl acetate for 20 seconds and air dried. The images were taken in a JEOL HEM 1010 microscope.

Data Analysis. The linear velocity of the recirculating electrolyte was calculated with the flow rate of the recirculation pump (L min^{-1}) and the column internal diameter (46 mm) (flow rate/column section). The charge produced in the conductive bed during the cronoamperometric assays was calculated by integrating the total current response over time and subtracting the baseline current (abiotic signal). The accumulated charge in the ME-FBR under open circuit conditions was similarly calculated from the corresponding discharge curves obtained after re-polarization of the anode. In this case the steady state current was subtracted. The theorical geometric area of the bed, used for the estimation of the electron storage capacity of the fluid-like anode, was calculated considering spherical particles of an average diameter of 0.8 mm.

Experimental Procedures

ME-FBR Discharging Assays of *G. sulfurreducens* at Different Oxidative states. Cells were harvested by centrifugation (6 min at 8000 rpm, 25 ºC) from the chemostat effluent. The pellet was washed and resuspended (final OD_{600}=1) in an anoxic solution (50 mM phosphate buffered medium and 50 mM KCl) under an anoxic atmosphere of N2. Five aliquots of 10 mL of the cell suspension were incubated in the following conditions: 1) buffered medium (reduced cells); 2) buffer with 42 mM fumarate; 3) a second buffered medium (reduced cells); 4) buffer with 8.3 mM iron citrate; 5) buffer bubbled for 5 minutes with an air flow with oxygen serving as the oxidizing agent. After completing these incubation assays and in order to remove the chemical oxidants from the medium, 3 mL of each of those aliquots were centrifuged (6 min at 8000 rpm, 25 ºC). Cells were then washed and resuspended in 3 mL of the anoxic buffered solution before adding them into the ME-FBR at the indicated times.

ME-FBR Discharging Assays of *G. sulfurreducens* with Different Cytochrome Content. Regardless of the conditions of the culture, cells were harvested (centrifuged for 6 min at 8000 rpm, 25 ºC), washed and resuspended in the anoxic buffered solution (final OD_{600}=1). 2 mL of the different suspensions were

consecutively added to the ME-FBR when the current reached the initial abiotic baseline.

ME-FBR Assays for Promoting Growth of *G. sulfurreducens*. The planktonic growth of electroactive cells of *Geobacter* was achieved by using the fluidized anode as the sole electron acceptor. Four ME-FBRs containing 58 g of glassy carbon particles (bed volume of 80 mL) were assembled under sterile conditions by autoclaving the elements of the system. For the ME-FBR-1, ME-FBR-2 and ME-FBR-4 the current collector used was a graphite plate whereas for ME-FBR-3 we used a platinum wire in order to minimize the use of the current collector as electron acceptor by the cells in the ME-FBR. The fluid-anode was polarized to 0.4 V in the system 1, 2 and 3. The ME-FBR-4 was maintained at open circuit potential condition in order to study the planktonic cell growth in the absence of an electron acceptor. A similar fifth microbial electrochemical reactor (ME-R) was built without fluidized anode and only with a flat and static graphite plate (4x4x0.5 cm) as anode (the recirculating flow was maintained) to study the planktonic growth under a fluidzed bed-free scenario. *G. sulfurreducens* batch grown cells were used as inoculum: $4.5 \cdot 10^9$ cells at exponential-phase were inoculated in the ME-FBR-1, for ME-FBR-2, ME-FBR-3, ME-FBR-4 and ME-FBR we used a suspension of 10^{10} cells at stationary-phase. The freshwater medium (0.55 L) contained 23 mM of acetate as the sole carbon and electron source. Samples were periodically removed from the liquid medium of the fluidized anode. Bacterial growth was measured by direct counting in a Neubauer chamber with the cells stained with the kit LIVE/DEAD as described previously. The planktonic growth for each sample was calculated by substracting the initial number of live cells (SYTO9 stain) observed right after the inoculation of the ME-FBR to the number of live cells observed for each sample.

ME-FBR Discharging Assays of *G. sulfurreducens* After Open Circuit Periods. A ME-FBR, assembled and inoculated with *G. sulfurreducens* as described above, was operated for 2 months. Acetate additions (10 mM) were performed when current was diminished or acetate was depleted. The medium was periodically amended and replenished with fresh anoxic media. The accumulation of charge in the bacteria was enhanced by maintaining the system at OCP for several periods of time under two different experimental scenarios: a) under either acetate starvation (non catalytic conditions) or b) under acetate in excess (catalytic conditions). The OCP of the anode during these periods was measured with a multimeter (Keithley Instruments, Model 2700) acquiring 1 measurement per second. After these biological-charging periods, the anode was polarized while chronoamperometric measurements were performed by recording the current every 1 second.

Assays with Insoluble Fe (III) Respiration of *G. sulfurreducens* Grown Under Different Conditions. Two of the ME-FBRs used for promoting the planktonic growth of *G. sulfurreducens* respiring the fluidized anode were used for testing the ability of those cells (ME-FBR-grown cells) for reducing ferrihrydrite. The electrodes of those reactors were disconnected and the current collector and the cathode removed from the medium. Two more ME-FBRs were assembled, filled with sterile medium, and desoxygenated, as previously stated. They were inoculated with a suspension of a total of $1.6 \cdot 10^{10}$ cells of *G.sulfurreduces* previously grown with fumarate serving as final electron acceptor (fumarate-grown cells). The total amount of cells inoculated in each reactor was ca. the same. A fifth ME-FBR reactor was used as a control in order to test the abiotic reaction. Ferrihydrite was added (final concentration ca. 6 mM) to each ME-FBR as electron acceptor, and samples were anaerobically collected periodically and incubated in HCl 2 N. In addition, a similar assay was performed but in sealed bottles in order to study the role of the fluidized conductive particles on the microbial iron reduction reaction. Thus, ME-FBR-grown cells (total amount of cells of *ca*. $4 \cdot 10^7$ cells mL^{-1}), from a new ME-FBR constructed and producing current, were transferred to 6 sealed bottles containing acetate and ferrihydrite (final concentration of 10 mM and 5 mM, respectively). Three of those bottles also contained 10 mL of glassy carbon particles. Samples were taken as described above. The Fe (II) production rates were calculated by susbstracting the average abiotic Fe (II) production rate during the entire assay to the values for the biological assays.

2.4 Results And Discussion

Discharging Of Plug-And-Play Geobacter Cells In The ME-FBR

We first used electroactive *plug-and-play cell*s for testing the response of a fluid-like electrode made of glassy carbon microparticles as part of a Microbial Electrochemical-Fluidized Bed Reactor (ME-FBR) (schematic shown at Supplemetary Fig. 2-1). When a suspension of cells grown in a chemostat under electron acceptor limitating conditions was added to the ME-FBR, an immediate increase in the current density was observed, indicating an effective extracellular electron transfer (EET) with the fluid-like anode. This electron discharge was found to be dependant on the incubation conditions of the plug-and-play cells before prior to inoculation into the ME-FBR (Figure 2-1.A). When a suspension of these cells was incubated with buffer, a total of 58 mC were collected. In contrast, when a suspension of *plug-and-play cells* was incubated with electron acceptors able to

withdraw electrons from the outermost membrane cytochromes (Esteve-Núñez *et al.*, 2008) (eg. Fe(III)-citrate or oxygen) then the current densities decreased and the EET was decreased up to 76 % (14 mC). Inhibition of current density or on the total electron discharge was not observed when the cells were preincubated in fumarate, a soluble electron acceptor (Butler *et al.*, 2006) that does not interact with the outermost redox elements involved in EET.

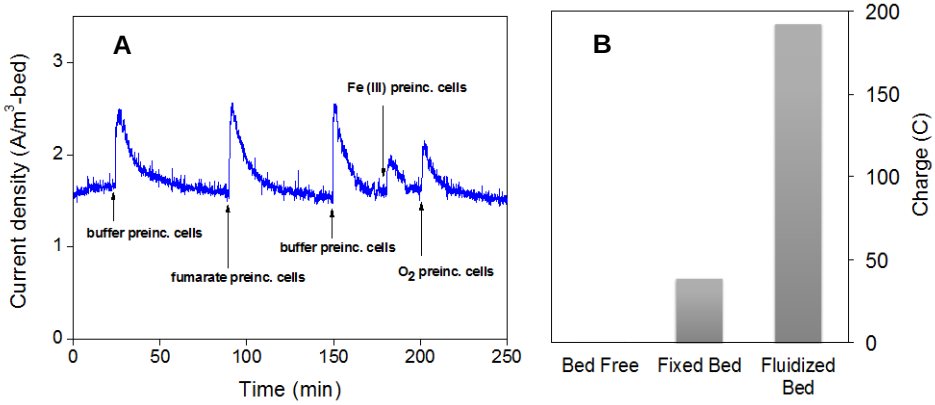


Figure 2-1: Electron discharge of the plug-and-play cells in the fluid-like anode. A. Current density produced on the ME-FBR as a result of adding plug-and-play cells preincubated under different conditions (linear velocity of 0.71 cm s^{-1}). B. Charge obtained under the different scenarios tested in the ME-FBR. The suspended bed condition was tested at a linear velocity of 1.19 cm s^{-1} in the presence of a current collector.

The electrical connection between these cells and the fluidized anode was also explored in motion, revealing an electron discharge 5-fold higher compared with a static and conventional system such as a fixed electroconductive bed. Furthermore, no charge was obtained in the absence of the bed of particles which eliminates any major role of the current collector for accepting electrons (Figure 2-1.B). Our results showed that the capacity for electron storage ($3 \cdot 10^{-16}$ mol e^- $cell^{-1}$) in plug-and-play cells was significantly higher (18-fold) than those estimated in previous studies based on the reduction of soluble electron acceptors for *Geobacter* (Esteve-Núñez *et al.*, 2008). Soret-peaks are classical signals in the absorption spectrum of cytochromes *c*. They typically shift due to the presence of oxidants like oxygen and Fe(III) (see Supplemetary Fig. 2-2), revealing an oxidation of the heme groups. The fact that Fe(III) or oxygen pre-treatments minimize the exocellular electron transfer to the fluid-like anode suggests that *c*-type cytochromes play a key

role in EET to the fluid-like electrode. This hypothesis was further confirmed when the capacity for the EET to the fluid-like anode was severely decreased by testing *G. sulfurreducens* cells with very low *c*-type cytochrome content (Estevez-Canales *et al.*, 2014). This cell physiology was achieved by growing *Geobacter* cells in the absence of iron, a condition which strongly downregulates *c*-type cytochromes (Embree *et al.*, 2014) (see Supplemetary Fig. 2-3).

Geobacter Growth Performing EET In The ME-FBR

Once planktonic cell's EET was successfully proven, the next challenge was to demonstrate that microbial growth with this fluid-like anode was occuring. Therefore, the growth of planktonic *G. sulfurreducens* was followed by measuring different parameters such as acetate depletion, current production, and direct cell counts. Our results revealed (Figure 2-2.A) earlier current production in the system inoculated with cells at the exponential growth phase (ME-FBR-1). The average planktonic cell density by day 11 was of ca. $6 \cdot 10^{7}$ cells mL^{-1} in the ME-FBRs medium, coinciding with the maximum current density value of 140 fA $cell^{-1}$ or an electron transfer rate of 125 fmol e^{-} $cell^{-1}$ day^{-1}. This value is similar to those reported elsewhere for the electron transfer rates to Fe-oxides (225 fmol $cell^{-1}$ day^{-1} (Caccavo *et al.*, 1994)). Assuming a value of $2.1 \cdot 10^{-11}$ g-dry weight $cell^{-1}$ for *Geobacter* species (Tang *et al.*, 2007) and a 43 % of protein content, the current densities produced varied from 2.5 to 14 mA $mg\text{-}prot^{-1}$, and the electron transport rates from 1.6 to 9 $\mu mol\text{-}e^{-}$ min^{-1} $mg\text{-}prot^{-1}$. These values are similar to those previously reported for *Geobacter* biofilm cells, indicating that the respiration rate of planktonic cells in fluid-like anodes is comparable to that of electrode-attached cells (Bond and Lovley, 2003; Marsili *et al.*, 2010). Coulombic efficiencies reached values over 91 % during the exponential current phase when the EET was at its peak. An average doubling time of 38 ± 8 hours was estimated for this stage, which is slightly higher than the values reported for growth of *G. sulfurreducens* with Fe-oxides (12-24 hours) (Bond and Lovley, 2003). When an identical system, inoculated with *G. sulfurreducens*, was operated under open circuit conditions (ME-FBR-4), no acetate consumption was observed and no planktonic cell growth was promoted (Figure 2-2.B). This proves that planktonic cell growth requires the fluid-like anode to be polarized so the current may circulate and bacteria can use it as a suitable terminal electron acceptor.

A cyclic voltametric analysis of the fluidized anode of ME-FBR-1 gave us more information about the electrode-bacteria interaction (Figure 2-2.C). The voltammogram obtained at the maximum current density production (black line)

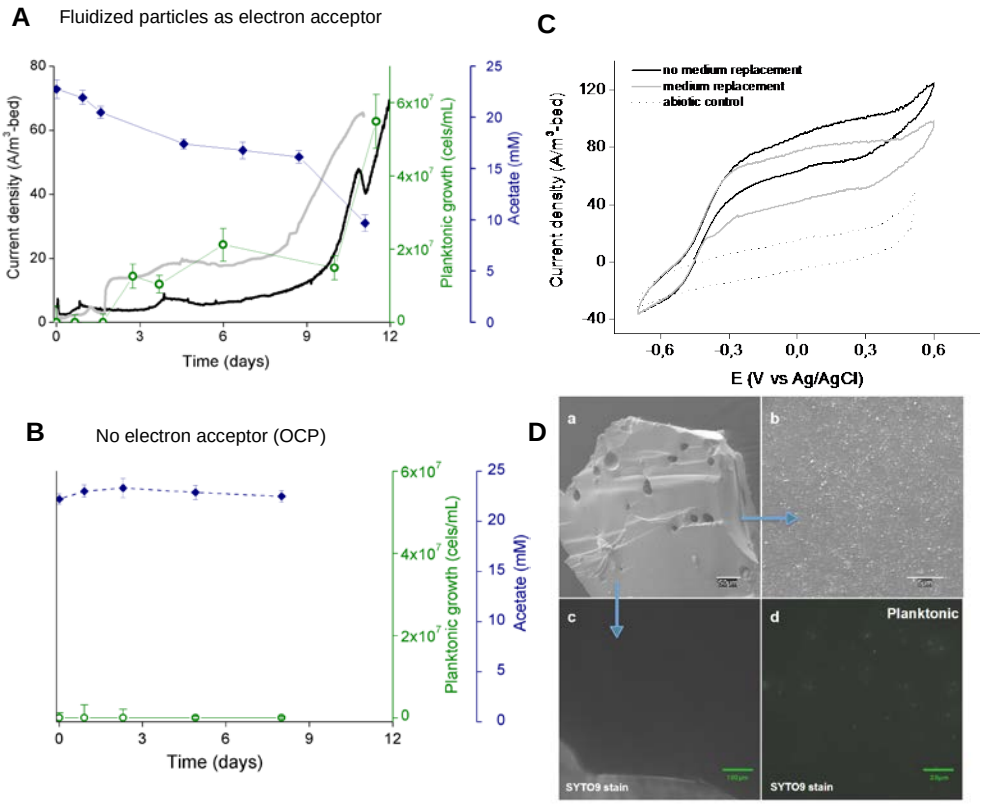


Figure 2-2: A: Growth of planktonic cells of *G. sulfurreducens* (○), acetate in medium (◆) and current density production in 2 independent reactors (ME-FBR-1 and ME-FBR-2) (— and —). All the values, except for the current density, are the mean of the two systems. B: Growth of planktonic cells of *G. sulfurreducens* and acetate in the medium in the ME-FBR-4 operated at open circuit potential (no electron acceptor available in the medium). C: Voltammograms at the time of maximum current in ME-FBR-1 at a rate of 5 mV s^{-1} (—), right after the 50 % of the medium was replaced by a fresh sterile one acetate added (—) and at a cell-free condition (------). D: Micrographs from SEM (a and b) and fluorescence microscopy (c and d) from the fluidized particles (a, b and c) and from the medium (d) of a ME-FBR with the bed serving as electron donor.

showed the same redox pattern as that obtained for *Geobacter* species (Katuri *et al.*, 2010; Richter *et al.*, 2009) with a major redox sites with a formal potential of -0.42 V and a minor one at 0.03 V (first derivative shown at Supplementary Figure 2-4). A media replacement with fresh sterile media generated a new voltammogram (grey line) with a lower oxidative current but the same formal potential. This suggests that the mechanism for establishing electron transfer between cells and the fluidized anode was the same but the rate of the bioelectrocatalysis was lower. We attribute this to the reduction of the planktonic cell density of electroactive bacteria and not to

the reduction of activity since the new medium (including 50 % fresh medium) contained sufficient acetate (> 7 mM) and nutrients. If cells in the ME-FBR were attached to a surface, either individually or by forming a biofilm, the current generation would remain unaffected by the medium replacement or even increased after providing fresh nutrients and waste removal (Bond and Lovley, 2003). Actually, when we operated the ME-FBR-3 with a polarized anode of fluidized bed of glassy carbon particles but a platinum wire as current collector (very low superficial area), we observed planktonic cell growth coupled to current production (Supplementary Figure 2-5). The surface exploration of the glassy carbon particles by scanning electron microscopy confirmed the absence of attached biomass in all our assays (Figure 2-2.D.a and b), demonstrating that the current observed was due to the interaction between the planktonic cells and the fluidized anode with no biofilm formation. Fluorescence microscopy imaging also confirmed that no biofilm was developed over the anodic particles (Figure 2-2.D.c) and revealed the presence of bacteria population in a planktonic state (Figure 2-2.D.d). TEM images showed that these planktonic cells grown in the ME-FBRs were viable and pili-free (Figure 2-3). The absence of pili was expected since this element is used for transporting electrons along biofilms in *G. sulfurreducens* and also for adhesion and attachment (Bond *et al.*, 2012).

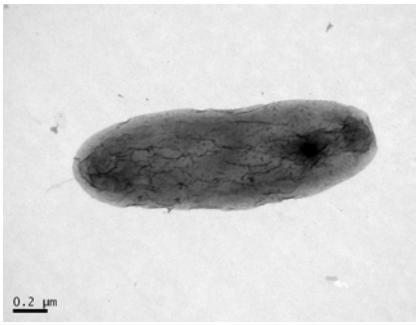


Figure 2-3: Planktonic *G. sulfurreducens* cell grown in a ME-FBR respiring the fluidized anode. TEM image of a single cell from the medium of a ME-FBR producing current coupled to acetate oxidation.

As a positive control, we tested a ME-FBR with no fluidized particles and a flat conductive surface as sole electron acceptor. The planktonic growth did not occur and the cells interacted with the electrode by forming a biofilm (see Supplementary Figure 2-6). This suggests that the promoting factor for the planktonic cell growth was the nature and fluidized state of the conductive particles that stimulates the existance of a direct and individual cell-particle interaction.

The viability of the planktonic interaction between cells and the fluid-like electrode was tested for over 2 months, in which multiple medium replacements and acetate additions were performed (see Supplementary Figure 2-7). During this experimental period, the redox coupling was sufficiently balanced for promoting microbial growth. This means that not only a ME-FBR can be utilized as an electron discharging element for microorganisms but also that the fluid-like anode may support microbial growth over a prolonged period of time. To our knowledge, this is the first time that bacteria like *Geobacter*, with an EET based on direct transfer, were cultivated under a planktonic mode by respiring an electrode. The process requires the microbial storage of electrons in the periods when the electron acceptor is not physically in contact with bacteria, until they are finally released as soon as cells interact with the fluid-like anodic particles. This electron storage capacity of *Geobacter* was hypothesized to be a novel mechanism, so-called *iron lungs*, for short-term energy generation in absence of a suitable electron acceptor (Lovley, 2008).

Discharging Assays Of G. sulfurreducens After Open Circuit Periods

The vast network of *c*-type cytochromes of *Geobacter* can function like a pseudocapacitor by accepting electrons from acetate metabolism when extracellular electron acceptors are not available (Esteve-Núñez *et al.*, 2008; Lovley, 2008). This phenomenon has also been shown for electroactive biofilms attached to graphite bars, which have the capacity for discharging the stored electrons with no limitation on the conductivity of the biofilm network (Schrott *et al.*, 2011).

The same charge-discharge phenomena was assayed with our fluid-like electrode over 2 months by performing cycles of disconnection (electron acceptor starvation period) and reconnection of the fluid-anode polarization. Those cycles were assessed under two different scenarios: under catalytic conditions with acetate as electron donor (turnover), and non-catalytic condition (non-turnover). At the time of disconnection, the open circuit potential (OCP) of the fluidized anode was measured. Under this environment, the OCP of the anode decreased to ca. 0.2 V in the first 100 min at non-turnover conditions (Figure 2-4.A). In contrast, when acetate was supplied to the medium the potential reached -0.46 V over this same period of time. This potential might be associated with the reduction of the heme groups responsible for the electron storage. When the fluidized anode was polarized back to 0.4 V, the collected charge actually increased with the time under OCP (Figure 2-4.B). In fact, the values were always higher in the presence of acetate, suggesting that the planktonic cells were able to accumulate electrons from acetate oxidation for

long periods of time and afterwards donate them to the fluidized anode. When the system was maintained at OCP for longer periods than 53 min, the collected charge tended to decrease, whereas the OCP, which was an indication of the generated reducing power, reached values as low as -0.54 V until 300 min. This discordance suggests that TEA-starvation for such long periods of time could be attributed to a reduction in the activity of the electroactive microbial population, or to the development of different pathways for consuming the excess of electrons (reducing power), i.e., in the form of hydrogen[1]. Maximum currents obtained at the cronoamperometric curves were also dependent on the time at OCP. The current leak, due to self-discharge processes, was estimated from the monitored open circuit potential drop and resulted to be of 0.55 % over 8.5 h and at a rate of 0.001 μV s^{-1}, outperforming the properties reported for the self-discharge of *Geobacter* biofilms.

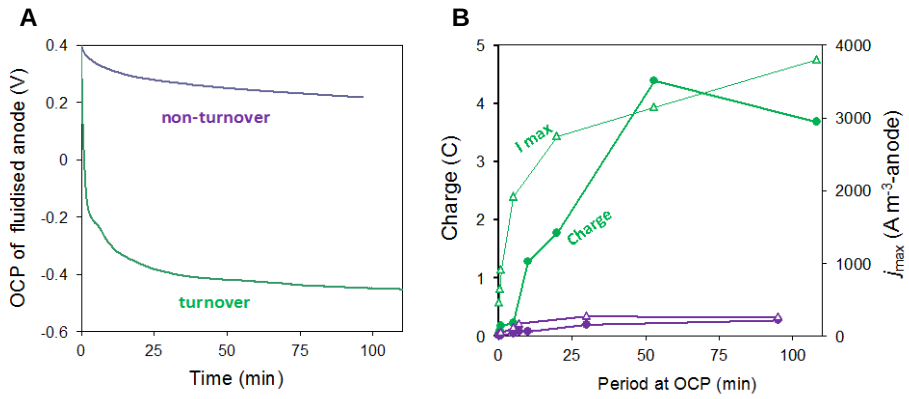


Figure 2-4: A. The evolution of OCP of the fluidized anode when current was disrupted under two different scenarios: non-turnover (purple) and turnover (green) conditions. The inset shows the OCP value over a longer period of time. B. Maximum current density achieved (j_{max}) (triangles), and charge harvested (circles) from the corresponding cronoamperometries when the ME-FBR was polarized after different periods at OCP under non-turnover (purple) or turnover conditions (green). The assays were performed at a recirculating velocity of 0.71 cm s^{-1}.

We observed that maintaining the cells under electron acceptor starvation for periods as high as 53 min allowed one to collect a charge of 4.4 C, which corresponds to a microbial oxidation of 57 µmol of acetate. This time is remarkable and shows that planktonic cells cultured in a ME-FBR can oxidize acetate in the absence of a TEA for longer periods than do electroactive biofilms employing classical electrodes as rods or plates (30 min) (Schrott *et al.*, 2011). In addition, the estimated maximum electron storage capacity of the ME-FBR in this experiment was

of $1.2 \cdot 10^{-8}$ mol electrons cm^{-2}, which is 10-fold the values reported for an standard electroactive biofilm of *Geobacter sulfurreducens* (Schrott *et al.*, 2011). We hypothesize that these electroactive planktonic cells might have developed more efficient pathways for storing electrons than biofilm cells do. These pathways could be associated to changes in the *c*-type cytochrome network for increasing the electron storage capacity since the respiration of the planktonic cells in our ME-FBR is supported on an uncoupled process of charge (from acetate oxidation) and discharge (electron transfer to the fluid-like anode in motion). These results suggest that there might be significant differences between biofilm-forming cells and planktonics cells when they interact with a terminal insoluble electron acceptor. The motion state of both the fluidized anodic particles and *Geobacter* cells may eliminate the need of the cell for building long-distance pathways along the biofilm for 'reaching out to touch' the electrode. By recirculating media, *G. sulfurreducens* cells are provided with 'artificial motility' that continuously conducts the cells towards the electroconductive insoluble electron acceptor also in motion.

Insoluble Fe (III) Respiration Of Geobacter Planktonic Cells Grown In The ME-FBR

This scenario, in which planktonic cells temporaly store electrons and finally release them onto conductive particles, resembles the scenario of *Geobacter* in its natural habitat. In such environments, *Geobacter* species are typically planktonic and the iron oxides are heterogeneously dispersed. When a source of iron is depleted, the network of *c*-type cytochromes acts as electron sink until an alternative source of electron acceptor is available, for instance Fe-oxides (Gescher and Kappler, 2014) or other microbial species able to accept electrons as part of a direct interspecies electron transfer (DIET) (Rotaru *et al.*, 2015; Summers *et al.*, 2010). Because of this similarity, we speculated the planktonic cells grown on fluid-like anodes could have common physiology with *Geobacter* cells grown at the earth subsurface. Actually, it has been suggested that the presence of planktonic *Geobacter* cells in the environment could be a sign of metal reduction activity (Gescher and Kappler, 2014). Thus, we tested the ability of the planktonic cells pregrown on fluid-like anode to reduce in motion insoluble iron oxides (ferrihydrate) under a open circuit potential operation (fluid-like anode cannot accept electrons) (Figure 2-5.A). In parallel, we performed the same assay but with cells previously grown with a soluble electron acceptor (fumarate). The first 22 hours of assay revealed that iron respiration rate of cells grown on fluid-like anode was 7 ± 0.1 fmol-Fe (II) $cell^{-1}$ min^{-1}. This value corresponds to 10-fold the respiration rate showed by fumarate grown-cells added to a reactor with fluidized glassy carbon particles

(0.7±0.5 fmol-Fe (II) $cell^{-1}$ min^{-1}) (Figure 2-5.B). Moreover, after a period of 4 days of incubation with the iron oxides, the total Fe (II) detected was 5-fold higher in the system with ME-FBR-grown cells (Figure 2-5.A). These results suggest that those *Geobacter* planktonic cells utilizing the fluidized anode as final electron acceptor developed strategies involved in the iron reduction pathway that were not expressed when cells grow planktonic by respiring a soluble electron acceptor like fumarate. Since *c*-type cytochromes were involved in the electron transfer from *Geobacter* cells to our fluidized anode, we hypothesized that they might also have a role as outer membrane proteins in the electron transfer either directly onto insoluble iron oxides, or to the fluidized particles at open circuit potential. In this last case, the fluidized glassy carbon particles would be acting as electron shuttles like quinones or humic substance do in sedimentary environments (Nevin and Lovley, 2002).

The next series of experiments were conducted to elucidate if the conductive particles (at OCP) had any role on the microbial iron oxide reduction. We performed short-term assays in which ME-FBR-grown cells were incubated with the Fe gel in sealed bottles in the presence and in the absence of the glassy carbon particlesFigure 2-5.C). Our results showed similar iron reduction rates of the ME-FBR-grown cells in than the ones observed in motion in the ME-FBR. No difference on the Fe(II) production was observed when glassy carbon particles were present in the media. This means that the metal reduction process was directly taking place, without mediation of the conductive particles.Our results show the existance of a common pathway between the reduction of the fluidized anode and the iron oxide particles. Althouth the mechanisms for iron oxides reduction in *Geobacter* species differ from that required for electrodes reduction, previous studies have observed the presence of shared elements in the electron transfer in current-producing biofilms and iron-reducing cells (Holmes *et al.*, 2006; Reguera *et al.*, 2006). Elucidation of the key proteins involved in the electron transfer from the planktonic cells to the fluidized anode requires further biochemical investigation and could provide insights into the mechanisms for electron transfer to insoluble Fe(III) oxides of *Geobacter* species, the most abundant iron reducing microorganisms in diverse sedimentary environments.

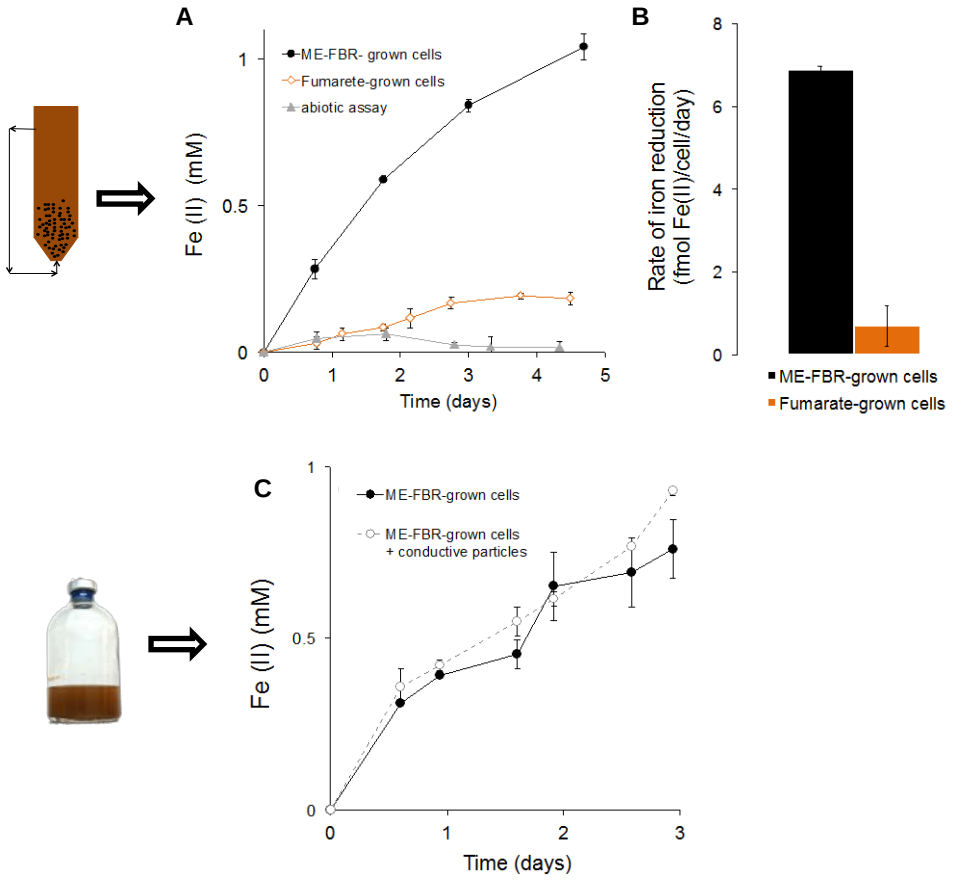


Figure 2-5: Fe-oxide reduction by fluidized-anode grown planktonic cells and by cells previously grown with fumarate. A. Fe (II) production from ferrihydrate reduction in motion in a ME-FBR at open circuit potential by ME-FBR-grown cells (n=2), by fumarate-grown cells (n=2) and in a cell-free media. B. Rate of iron reduction within the first 22 hours of cultivation of the cells with ferrihydrate in a ME-FBR. C. Fe (II) production from ferrihydrate reduction in sealed bottles with and without glassy carbon particles by cell suspensions of ME-FBR-grown planktonic cells (n=3).

2.5 Conclusions

We suggest an alternative to the paradigm of microbial EET where electroactive bacteria colonized an electrically conductive surface in order to directly transfer electrons to it. We demonstrate that *Geobacter* is able to directly transfer electrons previously stored in the cytochromes network to a suspended polarized electrode and that this interaction is able to support growth without the need of forming a biofilm. By promoting the interaction between electroactive planktonic cells

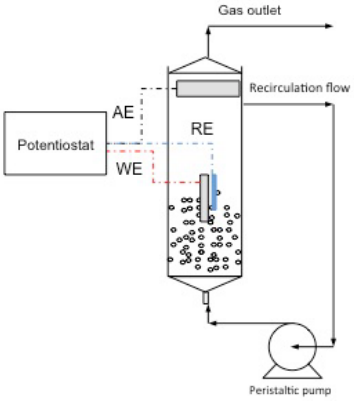
and a fluidized electrode made of microparticles in motion, every single cell is now contributing to current production. The construction of immobilized electrodes may favour biofilm development over the planktonic growth of electrogens, but by varying the surface and hydrodynamics of the electron acceptor, one can develop different scenarios with new strategies for growing electroactive bacteria. The simulated conditions in the ME-FBR, where *Geobacter* planktonic cells are wired to the conductive anodic particles in motion, could be representiative and useful for studying the interaction between insoluble electron acceptors and metal-reducing bacteria. A deeper analysis concerning the changes in cell physiology while performing continuous charging-discharging processes in the ME-FBR could provide further information that allows for increasing applications in this field.

Acknowledgments

This research was supported by the Center for the Development of Industrial Technology (CDTI), Spanish Ministry of Economy and Competitiveness, through the project ITACA (INNPRONTA program, CEN-20091005).

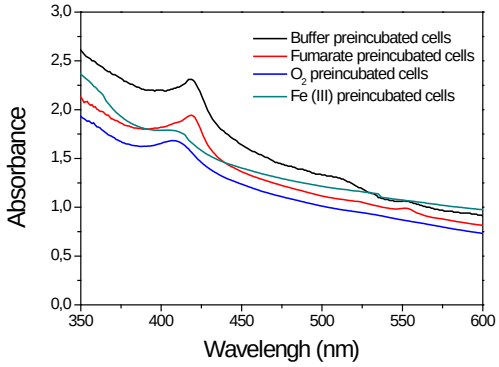
2.6 Supplementary Information

Schematic of the set-up of the ME-FBR.



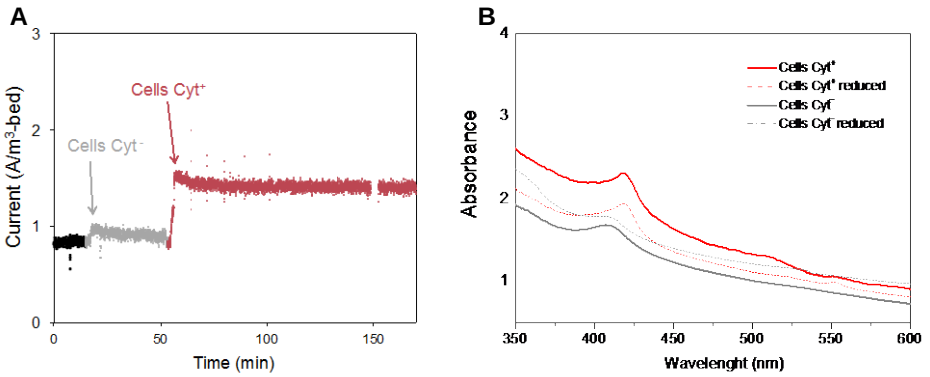
Supplementary Figure 2-1: Elements of the system set-up. The discontinuous lines indicate the electric connections, where WE stands for working electrode (current collector polarization), RE stands for reference electrode, and AE stands for counter electrode.

The redox state of the cytochromes in *Geobacter* cells suspension was followed by measuring the Soret-peak shift



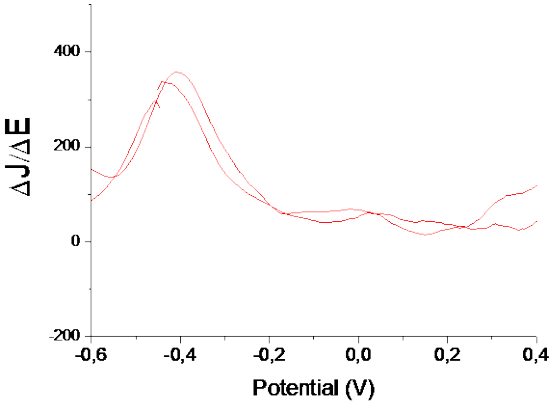
Supplementary Figure 2-2: Spectrums in the UV-range of the cells suspensions that were added to the ME-FBR. Redox state of cytochromes could be detected at 420 nm.

Electron discharge on fluid-like anode depends on cytochrome cell content



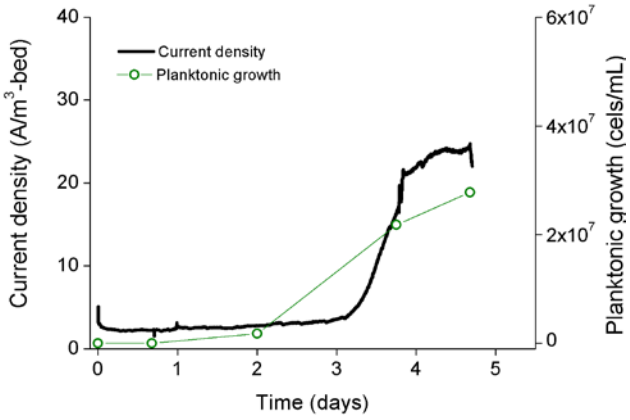
Supplementary Figure 2-3: A. Chronoamperometry showing how cell suspensions with different cytochrome content perform an electron discharge on a graphite-fluidized anode polarized to 0.4 V (linear velocity of 0.71 cm s^{-1}). B. Spectrums in the UV-range of the Cyt^- (low cytochrome content) and Cyt^+ (high cytochrome content) cells.

Redox sites for the cyclic voltammogram performed under turnover conditions in the ME-FBR



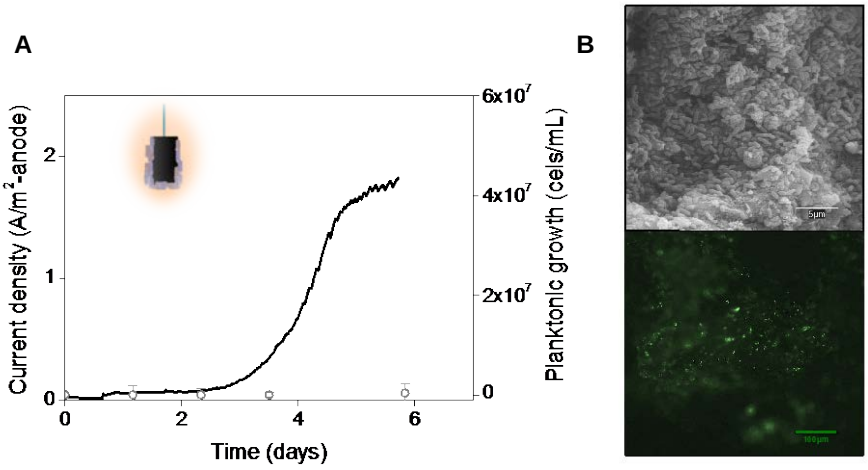
Supplementary Figure 2-4: First derivative of the current density (*j*) with respect the WE potential of the voltammogram from Figure 2.2.C of Chapter 2 (no medium replenishment).

Planktonic cell growth and current production in the ME-FBR with a platinum wire as current collector



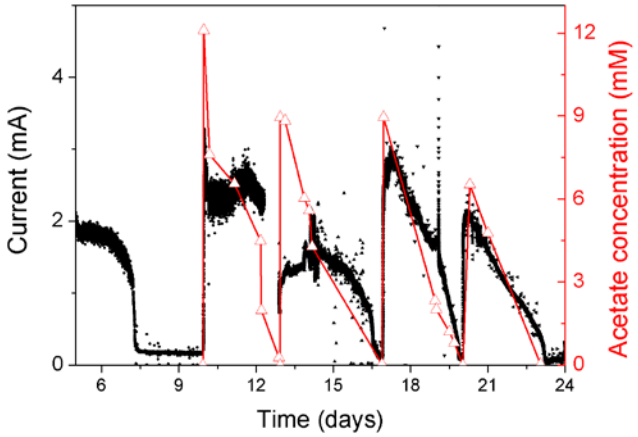
Supplementary Figure 2-5: Current density produced in the fluidized particles and the planktonic cell growth in the ME-FBR-3 medium.

No planktonic growth is observed in the ME-R if a flat electrode is the electron acceptor for *G. sulfurreducens*, a biofilm architecture is developed on the anode.



Supplementary Figure 2-6: A. Current density curve and *G. sulfurreducens* planktonic growth in a ME-FBR without bed and with a flat anode serving as sole electron acceptor. B. SEM and fluorescence micrographs of the attached biofilm developed in the flat anode immersed in the ME-FBR with recirculating flow.

Operational viability of the ME-FBR for culturing *G. sulfurreducens*



Supplementary Figure 2-7: Current produced with time as a result of successive acetate additions in the ME-FBR with a bed composed of glassy carbon particles and polarized to 0.4 V. Medium was replaced (1/3 of total volume) two times during the experimental period. The ME-FBR was operated at a linear velocity of 0.71 cm s^{-1}.

CHAPTER 3: Fluidized Bioanodes vs Non-Conductive Classical Fluidized Beds on the Treatment of a Brewery Effluent

This section has been redrafted after:

Sara Tejedor-Sanz[a,b], Hurt S. [c], César I. Torres[c], and Abraham Esteve-Núñez[a,b]. 2016. *Fluidized bioanodes vs non-conductive classical fluidized beds on the treatment of a brewery effluent*.

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Fluidized bioanodes versus non-conductive classical fluidized beds on the treatment of a brewery effluent

3.1 Abstract

In this study, we have compared the anaerobic digestion process versus the electrogenic metabolism on the treatment of a brewery wastewater in a fluidized reactor configuration under a continuous mode of operation. A microbial electrochemical fluidized bed reactor (ME-FBR), with a conductive bed made of activated carbon particles, was operated as a three-electrode electrochemical cell with the anode polarized to 0.2 V (vs Ag/AgCl). An additional classical anaerobic fluidized bed reactor was operated in parallel with the bed composed of biolite particles (biolite-M-FBR). Under the same scenario the ME-FBR outperformed the conventional FBR in terms of COD removal with more being achieved in the ME-FBR. Volatile fatty acids levels were always higher in the medium of the biolite-M-FBR, indicating that methanogenesis was properly coupled to the previous anaerobic digestion steps. In contrast, those acids remained at trace levels in the ME-FBR, suggesting that the electrogenic metabolism was perfectly coupled to the degradation of the complex organic substrates. Our results showed that the proportion of electrogenic metabolism highly depended on the organic loading rate applied. Low biomass growth was observed in the biolite-M-FBR as compared to the colonization found on the anodic particles of the M-FBR. Interestingly, the *Geobacter* cluster was highly enriched on the most inner layers of the biofilm formed on particles of the ME-FBR.

3.2 Introduction

Wastewater treatment technologies based on biological processes require a suitable electron acceptor to consume the electrons generated in the oxidation of organic waste. In this context, microbial electrochemical technologies (METs) represent a promising field based on the effective redox coupling between microbial metabolism and electrically conductive materials (Du *et al.*, 2007). The capacity of electroactive microorganisms to transfer electrons to an electrode (anode) or to use it as an electron source (cathode) offers a versatile platform with which to perform targeted redox reactions, i.e., oxidation of organic matter compounds or the

reduction nitrates from wastewater (Wang and Ren, 2013). Although urban wastewater (Brown *et al.*, 2015; Min and Logan, 2004) has been the most common biodegradable fuel tested in METs, industrial organic matter sources such as food industry residues have also been extensively tested in the last decade (Cercado-Quezada *et al.*, 2010; Çetinkaya *et al.*, 2015; Kelly and He, 2014a). From the very beginning,, brewery wastewater has received much attention due to the easy biodegradability of the organic components present in the effluents (mainly consist of sugars, soluble starch, ethanol and volatile fatty acids) are easy to biodegrade (Dong *et al.*, 2015; Feng *et al.*, 2008).

Due to the high COD content, anaerobic digestion is the classical technology used by brewery plants for eliminating organic matter, whereas nutrients are usually removed in an aerated tank. Both anaerobic digestion and microbial electrochemistry processes share common advantages: low sludge production, a low energy requirement, and the potential of recovery energy from the waste. However, a well known factor of anaerobic digesters is the instability of the process due to several factors, i.e., the difficulty of balancing acidogenesis and methanogenesis process as well as the presence of inhibitory compounds from wastewaters and sludge (ammonia, sulfide, light metal ions, heavy metals, halogenated organics) (Chen *et al.*, 2008). The slow growth and the high sensitivity of methanogens to different external agents can produce an accumulation of volatile fatty acids (VFAs) (acetic and propionic acids principally), provoking a decrease in pH. All of these vulnerabilities can produce perturbations causing the whole anaerobic digestion process to fail and reactor to be stopped.

In contrast, electrogenic metabolism is more robust than the one shown by methanogens. Electroactive microoganisms can work over a wider pH-range (2-10) (Badalamenti *et al.*, 2013; Carbajosa *et al.*, 2010; Zhuang *et al.*, 2010) and and even under halophile conditions (Badalamenti *et al.*, 2013; Carmona-Martínez *et al.*, 2013a; Miller and Oremland, 2008). The accumulation of VFAs in these systems is unlikely to occur since, under the presence of an anode acting as electron acceptor, VFAs are rapidly consumed in METs by electrogens producing an electron flux from the anode to the cathode. The quantity of this current flowing depends on the coulombic efficiency of the process. This in turn depends itself on the simultaneous competitive reactions occurring and the growth yield of the microorganisms. Methanogens and electrogens are direct and non-direct competitors for the electrons contained in VFAs. It has been reported that methanogens outcompete electrogens at high organic loads in METs (Sleutels *et al.*, 2016), whereas at high anodic

potentials and/or low substrate concentrations the electrogenic metabolism can be stimulated over that of methanogenic ones. However, a real application demands a capacity for treating high loads of organic matter in order to make the treatment process economically viable. The capacity of biological treatment systems is determined by the biomass amount (concentration, volume, etc) and the activity of the biomass. One of the designs able to achieve these two scenarios has been extensively used in the field of water treatment and is the fluidized bed rector. In this kind of configuration, the bed is fluidized by a controlled and uniformly distributed upward flow of liquid electrolyte, and when then minimum fluidization velocity is obtained, the electrode attains fluid-like behavior (Gupta and Sathiyamoorthy, 1998). These configurations are well known for biological wastewater treatment (Nicolella *et al.*, 2000) but the bed is typically composed of an inert carrier such as biolite in order to facilitate microbial adhesion. One of the newest applications of these systems is to merge them with a MET by using an electrically conductive bed, thus resulting in a microbial electrochemical fluidized bed reactor (ME-FBR), presented in Chapter 2. In this system, the fluidized bed functions as a polarized three-dimensional electrode with large specific surface to stimulate the degradation of organic matter by microbial process electrogenesis. The electrons from the oxidation of organic matter are transferred to the fluidized anodic particles, which are discharged in contact with a current collector. This design avoids some of the problems of using static biofilm-based electrodes, like the slow mass transfer between solution and active microorganisms, the low active surface area of the electrode, and the pH drop typically produced near the vicinity of the anode (Scott and Yu, 2015). A first approach employing this concept was reported using fluidized carbon granules as the anode in presence of artificial soluble redox mediators that were provided for enhancing microbial electron transfer (Kong *et al.*, 2011). Interestingly, direct electron transfer between *Geobacter* planktonic cells and a fluidized anode was observed in a microbial electrochemical fluidized bed reactor (see Chapter 2). In that system, a polarized fluidized anode made of glassy carbon particles served as final electron acceptor for *Geobacter sulfurreducens* and promoted the planktonic growth of this strain. This fact evidences the biofilm paradigm in bioelectrochemical systems. In contrast, a biofilm architecture can be achieved by a) increasing the complexity of the microbial community by shifting from pure *Geobacter* culture to a mixed community, and b) shifting the glassy carbon material to a more hydrophilic and irregular surface (i.e activated carbon) (Deeke *et al.*, 2015; Kong *et al.*, 2011). Although these biofilm-based systems still maintain some of the limitations related to the microbial electron transfer across the biofilm, they present some practical

advantages over the suspension culturing methods, for instance, it avoids the washing-out of the cells in the reactor.

In this work, we aim to demonstrate that a ME-FBR is a suitable bioelectrochemical technology for removing the organic matter from industrial wastewaters. We compare the performance of this system with one achieved using a classical fluidized bed digester made of non-conductive particles and operated under identical hydraulic conditions. Finally, we analyze the microbial community developed in these reactors.

3.3 Materials And Methods

Wastewater Description and Analysis. The wastewater samples used for all experiments were collected from the brewery plant Mahou-San Miguel in Alovera, Guadalajara (Spain), and stored at -4ºC until used. Wastewater samples were taken from the homogenization tank that fed the anaerobic digester of the brewery plant. This water came from previous coagulation treatment and pH-adjustment processes. Wastewater samples were analyzed according to the Standard Methods for the Examination of Water and Wastewater (Eaton and Franson, 2005). Samples were frozen prior to their analysis at -20 ºC. COD, total N (TN) and total P (TP) were measured with Spectroquant TR420 and Spectroquant Pharo 100 kits from Merck. Total SS were determined by vacuum filtration using AP40 90 mm filters from Millipore. The pH was measured with pH meter “pH 25” from Crison. Ammonium and nitrate were analyzed with a Metrohm Advanced Compact IC model 861 with two channels. Biogas composition was monitored using a Varian 3350 chromatograph equipped with a Porapack Q 80:100 mesh column and a thermal conductivity detector, using N_2 as carrier gas, a column temperature of 80 ºC, injector at 110 ºC and detector at 150 ºC.

Experimental Design. The ME-FBR was designed as previously described in Chapter 2. At the top of the column was placed a biogas collector hood and the reactor was sealed during all the experiments in order to maintain an anoxic environment. For fluidizing, a recirculation flow was drawn from the top section using a peristaltic pump (Heidolph 5006). The total volume of the reactor was of 0.68 L (including the recirculation tube and the bed volume). The anode consisted of a bed composed of 80 mL (43 g) of activated carbon particles (0.6-1 mm diameter, Aquasorb). A graphite plate (2x8 cm) that was vertically immersed in the fluidized bed was used as current collector. The cathode consisted of a RGV 2000 graphite

felt piece (10x7x0.6 cm) from Mersen placed around the inside perimeter of the flask. A Ag/AgCl 3 M KCl electrode (HANNA) was employed as a reference electrode. The ME-FBR was operated as a three-electrode electrochemical cell and the conductive bed worked as the anode polarized at 0.2 V (all potentials are reported versus Ag/AgCl electrode). The potentiostat used was a NEV3 Nanoelectra. The electrolyte velocity used during all entire experimental period was of 0.68 cm s^{-1}.

The non-electrochemical fluidized bed reactor (biolite M-FBR) was constructed with the same design as the ME-FBR but without electrodes and a bed of 80 mL of biolite (particles diameter = 0.25-0.32 mm, ρ= 1.25 kg L^{-1}, specific surface area = 0.6 m^2 g^{-1}). The electrolyte velocity used for this reactor was of 0.34 cm s^{-1}.

Both reactors were inoculated with 50 mL of sludge from a wastewater treatment plant. For acclimatint the biomass, the reactors were previously operated under batch mode by feeding acetate supplemented brewery wastewater, and with buffered medium (*ca*. during 20 days). Afterwards, these reactors were operated in continuous mode with increasing organic loads, and decreasing concentrations of bicarbonate buffer (75, 50 and 0 mM Na_2HCO_3). A peristaltic pump (Watson and Marlow 205S) was used for continuously feeding the reactors through a tube located at the height of the bed. The effluent outlet was located at upper part of the column. The organic load was varied either by supplying a wastewater with different COD concentrations or by increasing the influent flow rate. Table 3-1 summarizes the reactor operating conditions for each experimental period and their duration. A schematic of these systems for the continuous mode of operation is shown in Figure 3-1.

Table 3-1: Reactor operating conditions during the continuous mode period.

Duration (days)	COD influent (mg L^{-1})	HRT (h)	OLR (kg $m^{-3}_{reactor}$ d^{-1})	Feed
1-24	580	53	0.24	BWW+ 50 mM $NaHCO_3$
25-55	870	53	0.36	BWW + 75 mM $NaHCO_3$
55-89	1480	53	0.62	BWW
90-111	900	53	0.38	BWW
113-129	600	53	0.25	BWW
130-157	2100	53	1.15	BWW
142-159	1600	28	1.26	BWW
160-168	1690	21	1.73	BWW

FISH Analysis. The microbial community attached to the fluidized particles was analyzed with probe combinations (50 ng μL^{-1}) of either (i) Cy5-ARC915 to target most Archaea (Stahl *et al.*, 1991), Cy3-EUB338 or Cy5-EUB338 to target most bacteria (Amann *et al.*, 1990) and (ii) Fluo-GEO3-A, Fluo-GEO3-B and Fluo-GEO3-A to target *Geobacter* genera (Richter *et al.*, 2007). Probes were synthesized by IDT (Coralville, IA). All DNA was stained with 4′ ,6-diamidino-2-phenylindole (DAPI). Samples were immediately fixed in 4 % paraformaldehyde (PFA), pH 7.4, for 2 h at room temperature, followed by three sequential washes of 1X phosphate buffer solution (PBS) (1.44 g L^{-1} Na2HPO4, 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, and g L^{-1} 0.2 NaH_2PO_4). The particles were washed by submerging them in these washing solutions. We stored the samples at -20ºC in a 50:50 ratio (vol/vol) of ethanol to PBS until further processing. For hybridization, the stored samples were dehydrated in a graded series of 50, 80, and 100 % non denatured-ethanol solutions by soaking them in beakers for 5 min each, and left to air dry. Following this procedure, cells were hybridized with 50 ng uL^{-1} of probes for 3 h as described previously (Manz *et al.*, 1992) in hybridization buffer (0.02 M Tris, 0.9 M NaCl, 0.01 % sodium dodecyl sulphate (SDS)) with a formamide concentration of 30 % (vol/vol) at 46°C. After hybridization, the slides were submerged in a washing buffer (0.02 M Tris-HCl, 0.01 % SDS, 0.005 M EDTA, 0.1 M NaCl, and ddH_2O) at 48ºC for 20 min (Manz *et al.*, 1992). Specifically, 700 µL of NaCl was used in our washing buffer after utilizing a 35 % formamide hybridization buffer. The slides were then rinsed with distilled deionized H_2O, air dried prior to microscopic investigations, and stored at -20 ºC before imaging. Images were taken in a Leica TCS SP5 AOBS Spectral Confocal Microscope, equipped with four laser (405, Ar, Kr/Ar, and He/Ne) (405, 561 and 633) and 4 prism spectrophotometer detectors. The objective used was a HCX APO L U-V-I 40.0x0.80 (water). The software was the Leica Confocal Software for multi-dimensional image series acquisition. The software built 3D images from sequences of 18 images, each one with an interval of 2 μm. Images were processed using the software package ImageJ 2.0.0 and the estimation of the proportion of each of the microbial communities with respect to the total biomass was performed using the pixel counting tool.

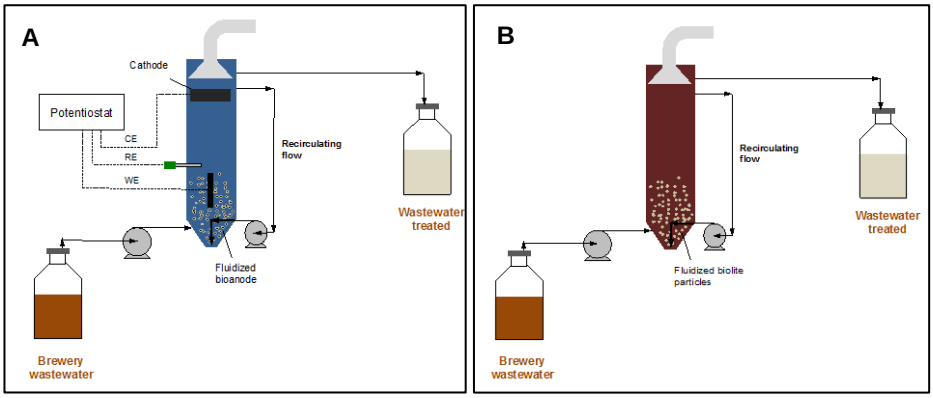

Figure 3-1: Schematic of the operated systems at continuous mode. A. The microbial electrochemical fluidized bed reactor. B. The biolite microbial fluidized bed reactor.

3.4 Results And Discussion

Treating Brewery Wastewater: ME-FBR Versus A Classical Fluidized Bed Reactor

The performance of the bioelectrochemical and non-bioeletrochemical fluidized bed reactors on the treatment of a brewery effluent was evaluated in terms organic matter removal.

From the beginning of the operation of the systems at continuous mode, the ME-FBR outperformed the classical fluidized digester with the biolite bed in terms of COD removal. As the organic loading rate (OLR) increased by enhancing the COD in the influent, the difference between both systems tended to be more significant (Figure 3-2.A). The removal of organic matter in the ME-FBR was always over 74 % whereas the biolite-M-FBR, with the influent having the highest COD feed, could not remove more than 22 % of this load. In spite of the enrichment stage under batch mode and at a continuous mode with low COD buffered influents, the biolite-M-FBR required a longer start-up stage for acclimation than did the ME-FBR. This could be associated with the double role of the electrically conductive bed: a) attachment, the chemical nature of the bed may influence on the bacteria attachment and biofilm development; and b) electron accepting nature, the bed may act as a respiratory substrate by accepting cells from cell metabolism. Biolite has been reported as a highly biocompatible material that promotes high biomass adhesion, and rapid start-up periods during the treatment of industrial wastewaters (Balaguer *et al.*, 1997). For this reason, we hypothesize that the nature of the material was not limiting biofilm development and biomass acclimation. Probably, the presence of an unlimited

electron acceptor, due to the electrode polarization, caused the enhancement in the microbial colonization of the anodic particles.

In addition to the higher performance, the ME-FBR showed a robuster behavour since the measured COD values of the effluent of the ME-FBR were more stable than those of the biolite-M-FBR.

Figure 3-2.B and C shows the evolution of the predominant acids in both reactors at two different OLRs. Interestingly, acetic acid, the main contributor to methane generation, was the short-chainn acid found at greater concentrations in both systems. Moreover, its concentrations increased in the two reactors as the OLR was enhanced. A similar trend, although not so marked, was found for the butyric acid. The levels of acids were always higher in the case of the biolite-M-FBR, for instance, at an OLR of 0.38 $m^{-3}_{reactor}$ d^{-1}, the total VFAs concentration in this configuration was 18-fold higher than the one observed in the ME-FBR. This accumulation of acids in the biolite-M-FBR revealed that methanogenesis was not being completely performed, suggesting that this reaction could be inhibited or not properly coupled to the acidogenesis and acetogenesis reactions. Regarding the possible inhibitory effects of the accumulation of acids in the biolite-M-FBR, the levels observed were below the ones reported to inhibit the methanogenic community (Wang *et al.*, 2009). The concentration of total VFAs at that OLR in the biolite-ME-FBR accounted for *ca*. 50 % of the total COD of the influent. However, the COD removal in the biolite-M-FBR was just 25 %, which suggests that not all the COD that remained in the influent was in the form of VFAs and more complex organic matter not degraded in the initial anaerobic digestion steps could be present as well. This would mean that the system was not only limited by the final stage of anaerobic digestion (methanogenesis), but also by previous reactions like hydrolysis.

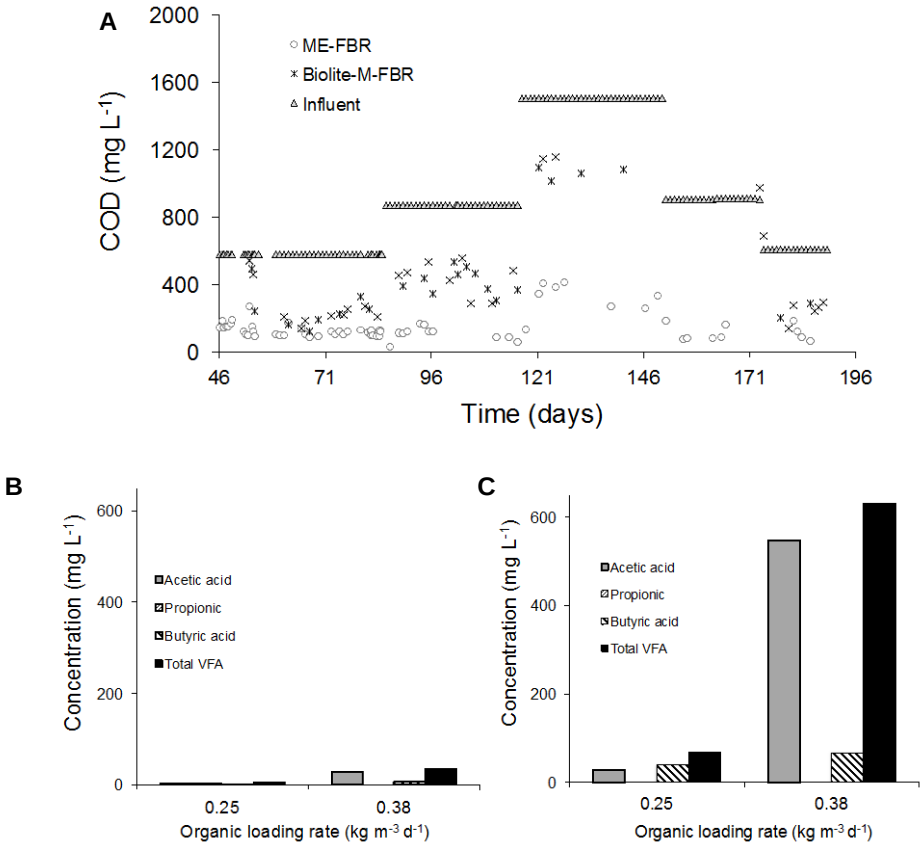

Figure 3-2: A. Chemical oxygen demand of the effluents of both the ME-FBR and the biolite-M-FBR, and of the influent (average value). B and C: Predominant volatile fatty acids measured in the ME-FBR (B) and the biolite-M-FBR (C) medium at 2 different organic loading rates.

In contrast, the bioelectrochemical system efficiently oxidized most of the acids formed from the complex of organic substrates probably due to the availability of an electron acceptor, or a faster acclimation, attachment and growth of the electroactive biomass as compared to methanogens.

Interestingly, the electrons harvested as current in the ME-FBR (OLR of 0.38 $m^{-3}_{reactor}$ d^{-1}) are an 85 % of those electrons contained in the accumulated acids in the biolite-M-FBR. This suggests that the difference in the performance of both systems could be mostly due to the difference between the methanogenic and the electrogenic pathways in order to consume the VFA. Probably, the VFAs in the biolite-M-FBR could be removed by providing an alternative electron acceptor to the system, either soluble (i.e. nitrate), or insoluble as an anode.

Performance Of The Systems Under A Fixed Bed Scenario

The impact of the fluidization of the EC bed was clearly detected when the systems were converted into a fixed bed scenario, by avoiding the recirculating flow. Interestingly, the performance of the ME-FBR severally decreased whereas the biolite-M-FBR capacity for removing COD was not significantly affected (Figure 3-3). When the recirculating pump is switched off, the bed is not expanded and the influent flow, which is fed directly to the bed zone, is not well distributed among all the particles. Under such a fixed bed operating condition, the fresh substrates, the metabolites, and the end products are slowly transported through the biofilm-electrode interface. In the particular case of the ME-FBR, this has an impact on the intracellular levels of $H^{\pm}$ because they are not consumed while respiring the electrode (just electrons are accepted by the electrode) as typically happens with other electron acceptors (*eg.* oxygen, fumarate, sulfate). This situation can cause an acidification of the biofilm as a result of the bioelectrochemical oxidation of organic matter and provoke depletion in activity of the electroactive community. This could be one of the reasons for which eliminating the fluidization state of the bed affected more negatively to the ME-FBR than to the biolite-M-FBR.

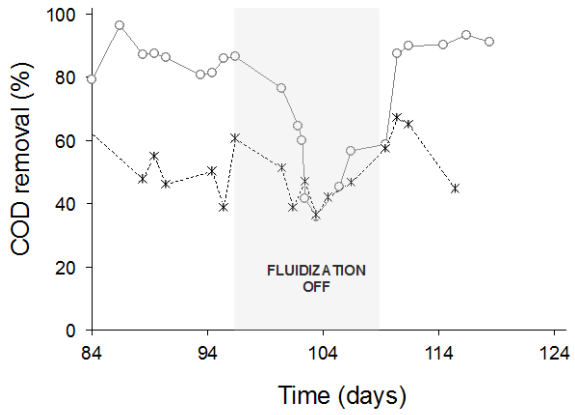

Figure 3-3: COD removal when the ME-FBR and the biolite M-FBR were operated as fluidized bed and as a fixed bed reactor.

Influence Of Organic Loading Rate On Reactor Performance

Next, we performed a serial of assays for testing the treatment capacity of each reactor at different organic loading rates In the case of the ME-FBR, the

removal efficiency increased as OLR was enhanced. Under the conditions tested, we did not achieve a maximum value of COD removal, which indicates that this system could further operate at ORLs higher than 1.7 kg $m^{-3}_{reactor}$ d^{-1} maintaining efficiencies over 96 % (Figure 3-4.A). The coulombic efficiency values for the ME-FBR, as expected, decreased as the system was fed with higher OLR although the current density values increased (Figure 3-5.A and B). This is the typical effect that has been extensively observed in bioelectrochemical systems using mixed cultures, and it is due to the outcompetition of other microbial metabolisms (eg. as methanogenesis) at high organic loads (Sleutels *et al.*, 2016). This means that at higher organic matter concentrations, a smaller part of substrate is used for current generation as compared to the part of substrate that is used for methane production. In our system, varying the HRT did not produce a washout effect in the reactor due the biomass attachment to the bed particles. In contrast, the organic load in the influent is probably the most influencing parameter over the CE.. Interestingly, the organic removal rate (ORR) increased linearly with the OLR over the ranges applied (Figure 3-4.C), indicating that the ME-FBR was capable of removing more substrate at higher OLRs .

Meanwhile, the biolite-M-FBR showed an opposite behavior when different ORL values were tested (Figure 3-4.B). The removal of COD in this reactor severally decreased when either OLR or flow rate were increased, which means that the system was being overloaded. The COD removal reached values as low as 24 % at an OLR of 0.7 kg COD m^{-3}-reactor d^{-1}, indicating that the system was not working properly. In spite of such a decreasing trend, In spite of such a decreasing trend, both system were able to remove a similar amount of organic matter at OLRs of 0.25 kg $m^{-3}_{reactor}$ $d^{-}1$ (Figure 2-4.C and Figure 3-4. D). Beyond this OLR, enhancing the OLR lead to lower substrate degradation rates. This result revealed a lower metabolic activity in the biolite-M-FBR that could be related to a low biomass growth or to the appearance of inhibitory process at high OLRs (accumulation of inhibitory compounds).

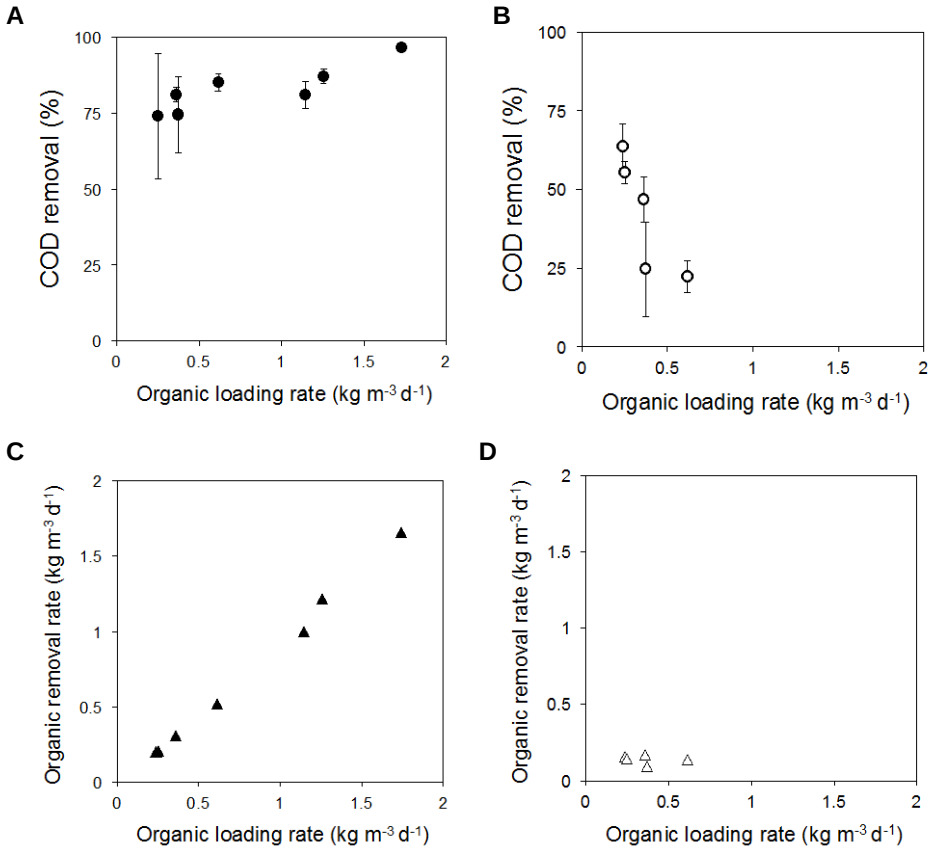

Figure 3-4:: COD removal (% and rates) versus organic loading rate for the ME-FBR (A and C) and the biolite-M-FBR (B and D).

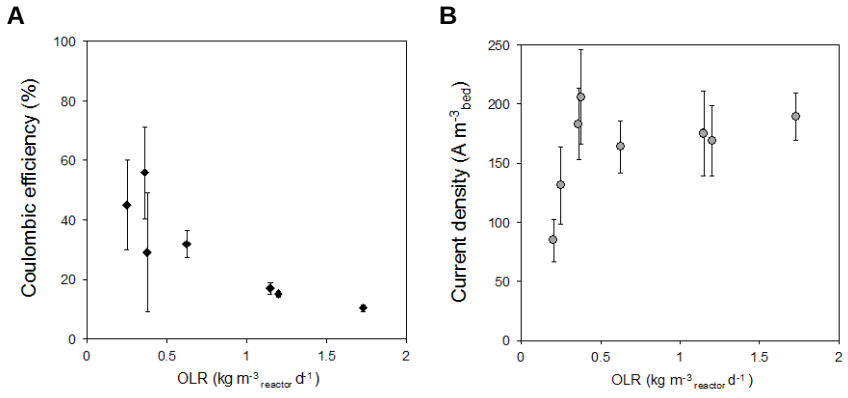

Figure 3-5: A. Coulombic efficiencies in ME-FBR at the different OLRs tested. B. Current density harvested in the ME-FBR at different OLRs.

Microbial Colonization Of The Particles

The differences in the performance of the ME-FBR and the biolite-FBR could be due to the different quantity and nature of the microbial population in the reactor. Thus, we proceeded to qualitatively study the colonization of the particles.

After 70 days of operation (counting both batch and continuous stages), the differences on the microbial colonization were highly visible as can be observe in Figure 3-6. The biofilm covered the big pores of the activated carbon particles in the ME-FBR by day 70, whereas the images from day 240 revealed a thicker with a high content of EPS and matrix in most of the particles surface. This biofilm had a high density of biomass, which is typical from systems exposed to shearing stress. It has been observed that the thickness, structure and even the metabolism of the biofilm in these kinds of systems are highly correlated to the detachment force (Chang *et al.*, 1991; Liu and Tay, 2001). At high shear stress, the growth yield is lower while the catabolic activity is enhanced (Liu and Tay, 2001), both desirable situations for the performance of our ME-FBR in order to achieve high coulombic efficiencies.

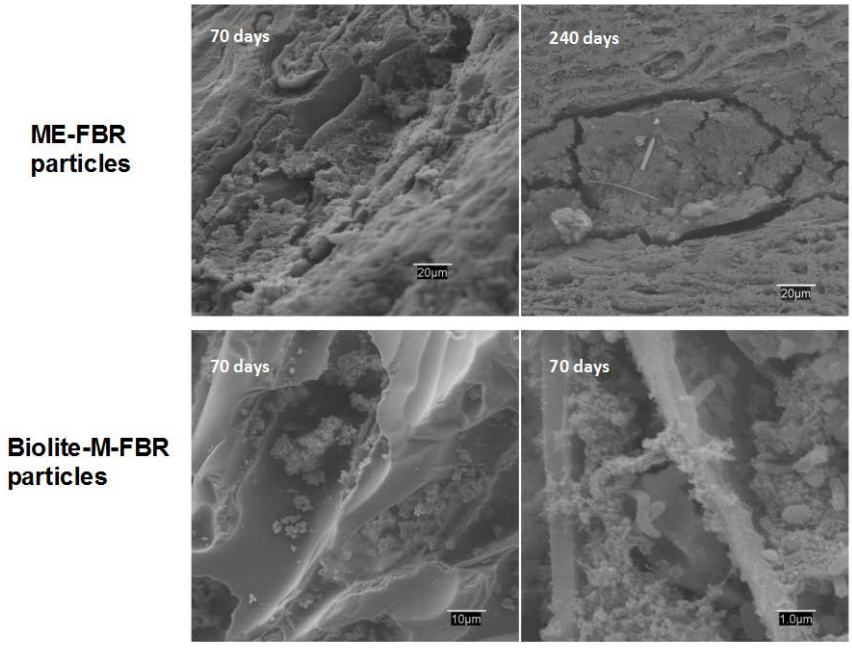

Figure 3-6: SEM images of the colonization on the particles of the ME-FBR and of the biolite-M-FBR after 4 months of operation.

The morphology of the wild biolite particles showed a laminar structure with internal cavities. In contrast to the scenario found in the activated carbon particles, the biolite particles were barely colonized aafter 70 days (Figure 3-6). SEM analysis of the cavities of the particles revealed the formation of aggregates of cells between the different layers of material rather than a mature biofilm. The difference in biomass growth was notable by day 70 could be the reason of such variation on the treatment efficiencies found between the two reactors.

Further FISH-based characterization of the electroactive biofilm developed on the activated carbon particles of the ME-FBR showed a partial coverage of the surface of the particles (Figure 3-7). The estimation from DAPI staining a microbial colonization of *ca*. 40 % of the surface (bright field). The colonization was heterogeneous since there were bare spaces of the surface without biomass attachment. Almost 50 % of the total biomass was composed of Eubacteria domain. Interestingly, the images showed a relative high abundance of *Geobacter* species (green) in the biofilm of the polarized particles (Figure 3-7). This is a strong indication for the presence of electroactive metabolism mainly performed by *Geobacter* strains oxidizing the short-chain acids and donating the resulting electrons to the fluidized anode. This result is consistent with the observations found in microbial electrochemical systems treating real wastewaters from the food industry, in which the anode present a high proportion of *Geobacter* species (Blanchet *et al.*, 2015; Kiely *et al.*, 2011). The relative high abundance of microbial species like *Geobacter*, the model microorganisms in microbial electrochemistry, suggests that the polarization of the conductive particles highly determined the microbial diversity in the biofilm as well as the formation of the biofilm. Because of this, we speculate that the activated carbon particles were a better carrier for microorganisms than the biolite particles because they acted as a terminal electron acceptor and therefore constituted a fundamental metabolic substrate for microorganisms.

The confocal microscope analysis (Figure 3-8.A and C) revealied a biofilm thickness (*ca*. 10 μm) that resulted relatively low compared to the ones previously observed in either fluidized bed reactors or in microbial electrochemical systems based on standard electrodes (Jana *et al.*, 2014; Liu and Tay, 2001). Actually, the most internal layers of the biofilm were mainly composed of *Geobacter* species, whereas the outermost zones were colonized by alternative species of bacteria (Figure 3-8.A). This suggests that *Geobacter* species in the ME-FBR could be responsible for the direct and ultimate transfer of electrons to the anode, either coming from their own metabolism, or from other microorganisms able to perform

interspecies electron transfer (Lovley, 2011; Rotaru *et al.*, 2014). The microbial community analysis also revealed the presence of species of Archaea domain under high OLR (Figure 3-8.B and C) (*ca*. 50 % of the total biomass, see Supplementary Figure 3-1) what is consistent with the low coulumbic efficiencies typically reported in microbial eelctrochremical systems when methanogens are present. Future studies focused on the dynamics of the microbial communities as a function of several system parameters such as the OLR, the electrochemical potential of the anode or bed expansion would bring useful information for optimizing the bioelectrochemical performance of these systems.

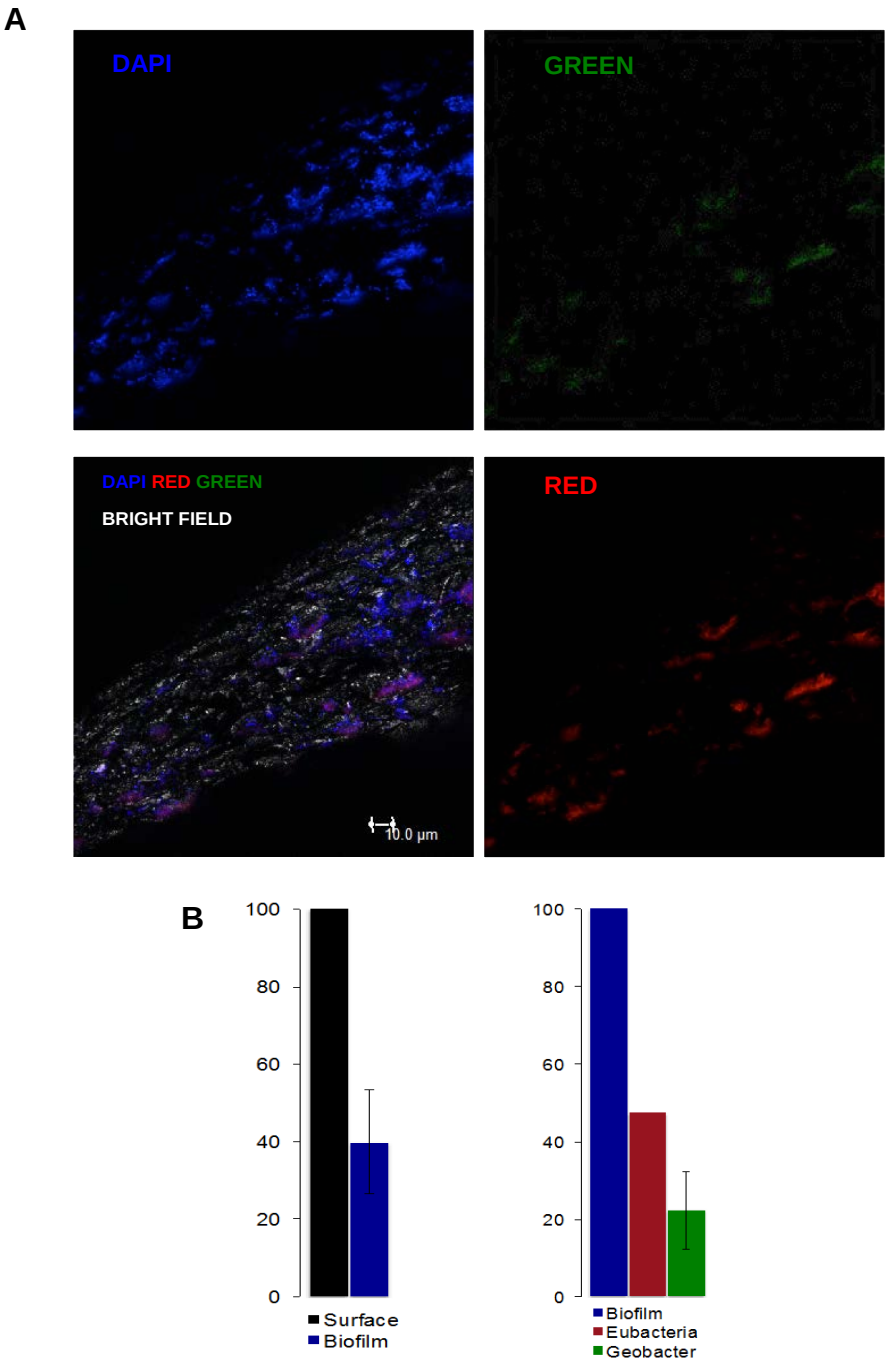

Figure 3-7: FISH experiments on the polarized particles of the ME-FBR**.** A. The blue signal corresponds to the DAPI stain (all nucleic acids), the red signal corresponds to the *Eubacteria* probe, the green one to the *Geobacter* cluster, while the white signal corresponds to the surface of the particle. B. Relative abundance of each of each stain estimated from at least 2 sequences of images taken for each sample.

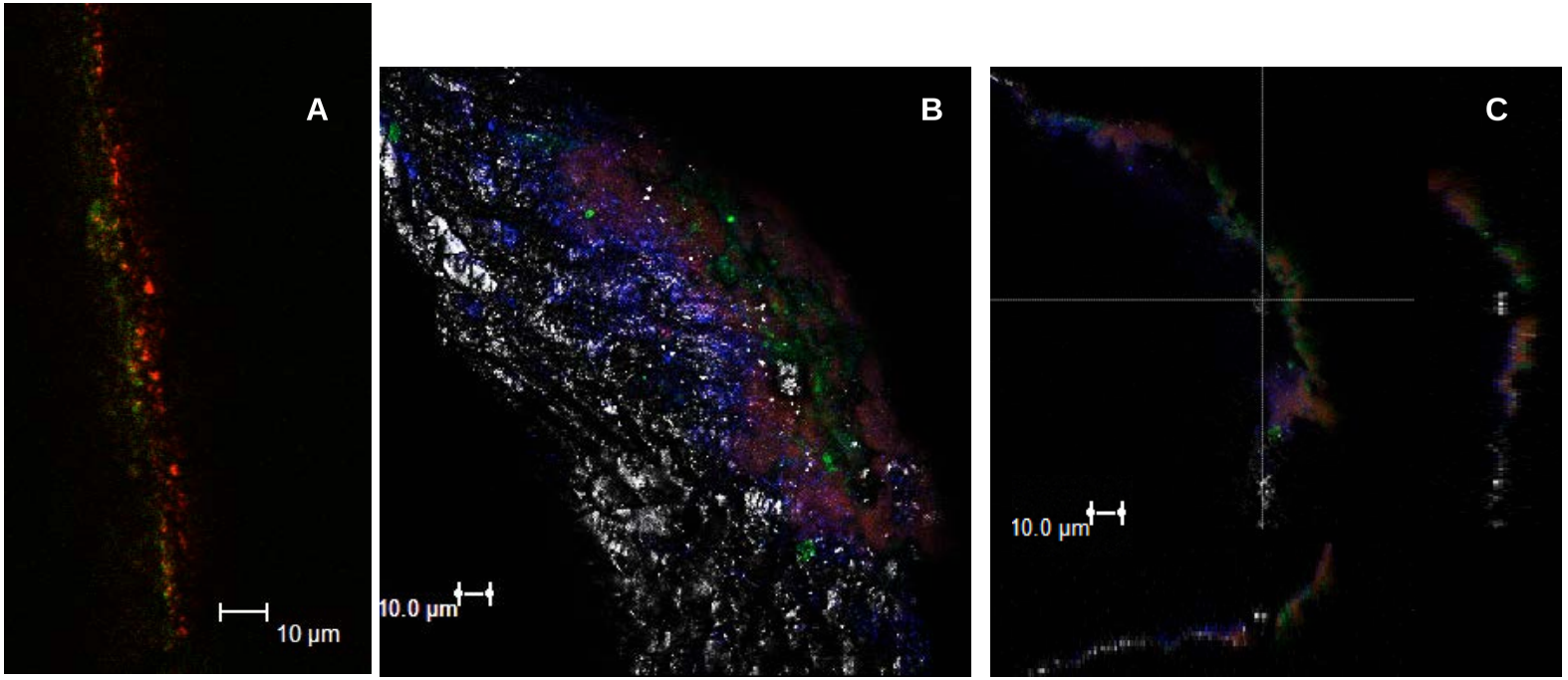

Figure 3-8: FISH experiments on the polarized particles. A. Section of a particle composed of the two signals: the green one corresponds to *Geobacter* cluster and the red one to Eubacteria. The zone located at the left of the image corresponds to the inner part of the particles, whereas the right side on black is the exterior zone. B. 3D Image projected from a sequence of images (sections were taken at 2-µm interval) of the biofilm formed over the surface of the particles (bright field). The green color corresponds to Eubacteria, the red color to the Archaea probe, and the blue signal to DAPI. C. Sections of the biofilm from image B in the 3 dimensions.

3.5 Conclusions

In this study we demonstrate that a fluidized bed bioreactor with a bed of polarized conductive particles outperforms the classical fluidized bed bioreactor for treating brewery wastewater by means of using the bed as terminal electron acceptor and not just as growth support for microbial growth. During the experimental period (170 days), the ME-FBR was able to remove up to a 60 % more COD than the biolite-M-FBR, which showed a lower performance as OLR was increased. In contrast, the ME-FBR was able to treat the brewery effluent further than at an OLR of 1.7 kg COD $m^{-3}_{reactor}$ d^{-1}. We attributed the better performance of the ME-FBR to a faster growth and colonization of the fluidized particles due to the respiratory role from the electrically-conductive bed..

In addition, our results showed that the proportion of electrogenic metabolism was highly dependant on the OLR applied. For real applications regarding brewery wastewater, there is low chance of operating this kind of reactor al low OLRs; so thus, at high OLRs, methane is likely to be the final product. Thus, it becomes necessary to perform more research on methods of stimulating the degradation of the simple organic matter by microbial electrogenesis rather than by methanogenesis. Regarding the economical sustainibility of the treatment, in a ME-FBR, there is an opportunity for recovering the H_2 produced at the cathode as an added-value product. The practical viability of this technology relies on both maximizing the recovery of this energetic vector and minimizing the energy demand of the power source.

In this study, we have shown the treatment of a brewery wastewater in a ME-FBR as a proof of concept. However, for developing a scalable prototype, each of the elements, parameters and the whole design of this system should be properly studied and optimized. We also highlight the importance of analyzing these systems from a chemical, electrochemical and microbiological point of view all at the same time.

Acknowledgments

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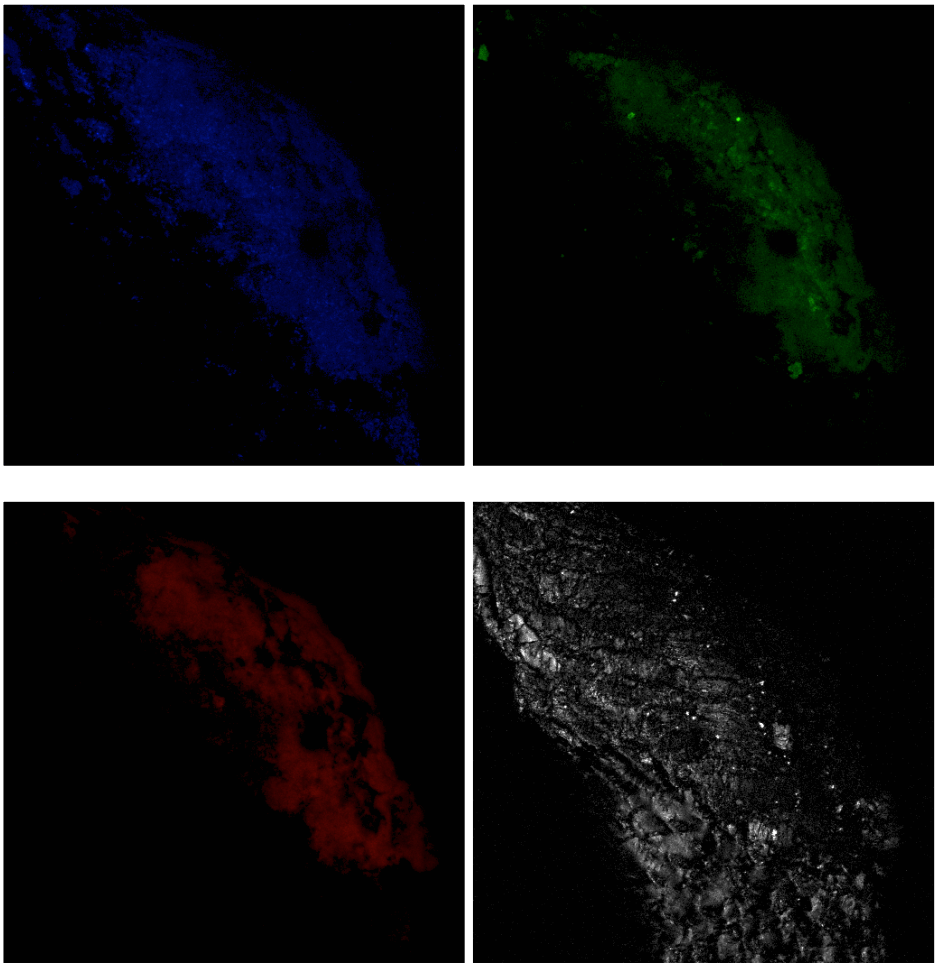
3.6 Supplementary Information



Supplementary Figure 3-1: The blue signal corresponds to the DAPI stain (all nucleid acids), the red signal corresponds to the *Eubacteria* probe, the green one targets the *Archaea*, while the white signal corresponds to the surface of the particle.

CHAPTER 4: Complementary Technologies to ME-FBRs for Achivieng a Complete Wastewater Treatment

CHAPTER 4

Part I: Integrating a Microbial Electrochemical System into a Classical Wastewater Treatment Configuration for Removing Nitrogen from Low COD Effluents

This section has been redrafted after:

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Integrating a Microbial Electrochemical System into a Classical Wastewater Treatment Configuration for Removing Nitrogen from Low COD Effluents

4.1 Abstract

The scaling-up process of METs may require an initial investment for constructing completely new infrastructure. In contrast, adapting METs to equipment already present in WWTP can be an attractive alternative to accelerate their implementation. In this study we evaluated the viability of adapting a classical oxic-anoxic chamber system to a membrane-free microbial electrochemical system in order to remove both nitrogen and organic matter. We simulated this configuration on a 22 L reactor of two chambers in absence of any separation membrane. The working electrode acted as electron source for denitrifying microorganisms and was placed in the first chamber. The system was able to support the nitrifying activity without external aeration and at oxygen levels below 2 mg L^{-1}. The influent, a synthetic media with ammonium as the sole nitrogen source, was fed at COD/N ratios from 2 to 4. Up to 19 g NO_3^--N m^{-3}-TCC day^{-1} were reduced at a COD/N ratio of 4, with a denitrification efficiency of 93 % and a nitrogen removal of 81 %. The system´s capacity for nitrifying and denitrifying was strongly dependent on both the COD/N ratio and the working electrode potential. A massive sequencing study revealed the greater abundance of such denitrifying genera as *Opitutos*, *Methyloversa* and *Zoogloea* at the cathode. Nitrifying genus as *Nitrosomonas* and *Nitrospira* were found in the reactor, the latter being enriched at the anode. In this study we demonstrate that the classical configuration of activated sludge systems can be turned into a MET to treat a wastewater. We suggest implementation of this air-free hybrid configuration in WWTP as an alternative method to remove nutrients from effluents with low levels of organic matter.

4.2 Introduction

Biological nitrogen removal is widespread, but the classical nitrification/heterotrophic denitrification process involves two major problems associated with wastewater treatment today: the large operational cost to aerate the nitrifying system and the need of electron donors (typically organic matter) for denitrification. In addition, the sludge produced from the heterotrophic aerobic metabolism needs to be properly managed, treated and disposed of, which

increases the operational costs. The requirements of new environmental legislation on municipal and industrial wastewater treatment have driven researchers to find more efficient technologies for wastewater treatment that minimize both the energy demand and the final waste while reusing the by-products generated. In this regard, new lines of research focus on alternative forms of microbial metabolisms (*e.g.* anammox (Mulder *et al.*, 1995) and microbial electrochemistry (Lovley, 2006)) while others try to optimize the already existing configurations. Modern WWTP dealing with high loads of organic matter typically operate under anaerobic conditions, with low operational costs and limited sludge production, while generating biogas as a reusable energy source (van Lier *et al.*, 2001). However, these technologies require the implementation of post-treatments, like serial aerated and anoxic tanks, or an anammox reactor, for the removing of nutrients, such as nitrogen (Chernicharo, 2006). Another possible configuration is the partial aerobic nitritation to nitrite followed by an anaerobic ammonium oxidation to dinitrogen gas, which is economically more viable than nitrification/denitrification. The limitations for implementing this system are the lack of practical experience on treating digesters effluents, the long start-up periods needed for enriching a reactor with anammox bacteria due to the low growth rate of these microorganisms and and the low amounts of biomass achieved inside the reactor (Fux and Siegrist, 2004). One of the newest and most promising technologies discovered over a decade ago is the use of electroactive bacteria, which have been extensively reported as having a large potential for wastewater treatment (Borjas *et al.*, 2015; Du *et al.*, 2007; Modin and Gustavsson, 2014; Rozendal *et al.*, 2008). METs have the potential of removing a wide range of organic compounds through the use of microorganisms that use an anode as their terminal electron acceptor (Jung and Regan, 2007). Likewise, electroactive bacteria can also use a cathode as electron donor for reducing substrates like nitrate, nitrite, sulphate, CO_2, tetrachloroethane, etc (Clauwaert *et al.*, 2007; Coma *et al.*, 2013; Strycharz *et al.*, 2008). Regarding the nitrogen removal in METs, several studies have investigated the fundamentals of this process and new designs have been proposed for simultaneous aerobic nitrification/bioelectrochemical denitrification (Sayess *et al.*, 2013; Virdis *et al.*, 2010), or in separate steps (Yan *et al.*, 2012a). Bioelectrochemical denitrification presents several advantages compared to the classical heterotrophic denitrification, for instance, the unlimited electron source of a cathode (supplied by an electric flux) while avoiding the need for the addition of external organic matter. Since the electron flux through the METs electrodes can be tuned, fine control over the rate of the reduction reactions can be performed in these systems so that treatment requirements can be achieved. All of these features make a microbial

electrochemical system a potential alternative for removing nitrogen from wastewaters with low organic matter content, or even from groundwater (Pous *et al.*, 2013; Tong and He, 2013). The scalability of METs in wastewater treatment plants is the main challenge in this innovative field. The success of this process requires optimizing the microbial reactions and minimizing the energy demand of the system, to reduce the electrochemical losses (Rozendal *et al.*, 2008). Another factor for implementing METs in WWTP is the reactor design and the cost of the initial investment. The fact that some aspects of a novel technology are still not well understood at lab scale can be a detriment at the time of performing real experiences with up-scale prototypes. In this regard, it can be economically advantageous to reuse already constructed designs to assess, at a large scale, novel technologies that otherwise are confined at the lab scale. Among the infrastructures that may become obsolete within the next decades, the classical 2 chamber activated sludge configuration may be the first candidate.

In this study we have investigate the treatment of low organic matter wastewater for removing nitrogen and carbon in a configuration that simulates a typical activated sludge reactor from a WWTP but using an air-free microbial electrochemical system operating in the electrolysis mode.

4.3 Materials And Methods

Reactor Set-up. The reactor consisted of a methacrylate vessel of 380 x 290 x 238 mm. It was divided in two compartments by a vertical plastic separator. The total working volume (TRV) was of 22 L. One of the compartments occupied 1/3 of the total volume and the other 2/3. The separator (290 x 238 mm) had a 20 x 50 mm hole at the bottom in order to let the media circulate through it from one compartment to the other when the system operated at continuous mode. Each compartment was mixed with a vertically rotating stirrer. The effluent outlet port was located in the second compartment, above the reactor liquid level. The effluent was directed to a rectangular-shape sedimentation unit (14 L) in order to collect the sludge produced.

The working electrode (WE) consisted of a 450 x 200 mm U-shaped carbon fiber cloth (Resinas Castro, 450 g m^{-2}) consisting of a continuous structure of 2 faces. The counter or auxiliar electrode (AE) was made of a carbon fiber cloth of 230 x 230 mm. A grade 1 titanium mesh was used as structural element and current collector for each electrode. The WE was placed in the smallest compartment with a Total Cathodic Compartment (TCC) volume of 7.33 L, and the AE in the other (parallel to the bottom wall of the reactor and in contact with air). Two reference

electrodes of Ag/AgCl 3M KCl sat. (HANNA) were used: one located in the vicinity of the WE, and the other one close to the AE). The WE was polarized with a Nanoelectra Nev4 potentiostat when the system worked under potentiostatic mode as a 3-electrode MEC. We used an μAutolab II to perform the galvanostatic assay (2-electrode configuration).

The reactor media (RM) contained 0.07 g L^{-1} NaCl, 0.02 g L^{-1} $CaCl_2 \cdot H_2O$, 0.01 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.08 g L^{-1} K_2HPO_4, 0.02 g L^{-1} $NaHCO_3$ and 0.1 mL L^{-1} of a mineral stock solution and a vitamin stock solution as previously described (Geelhoed and Stams, 2011). The inoculum consisted of 2.5 L of the liquor from an activated sludge reactor from a WWTP at CENTA (Carrión de los Céspedes, Sevilla, Spain). The media from the second chamber (2C) (simulating the oxic zone of an activated sludge system) was recirculated to the first chamber (1C) (simulating the anoxic zone, where denitrification is performed) through a peristaltic pump (Heidolph PD5006). The internal recirculation flux was 2-fold the influent flow at continuous mode (*ca*. 9.2 L d^{-1}). The influent was synthetic water resulted from a concentrated solution (25-fold) of the RM described above, supplemented with acetate and ammonium (see Table 4-1), and diluted with tap water just before inlet port. The influent was fed continuously with a peristaltic pump (Heidolph PD5006) into the first compartment of the reactor. Samples were taken periodically from the two chambers. The effluent samples were taken from the second chamber close to the outlet port. The reactor was operated at room temperature (from 20 to 25 ºC).

Measurements and Analyses. Nitrate and nitrite were measured in a Dionex DX120 Ion Chromatograph equipped with a conductivity detector, a cation suppressor and an IonPac 4 × 250 mm AS9-HC column. Ammonia was measured in a Metrohm 861 Advance Compact IC equipped with a METROSEP C3 250 column of 4 mm x 250 mm. The soluble organic matter in the effluent was measured in terms of Chemical Oxygen Demand according to the Standard Methods (Eaton and Franson, 2005). The dissolved oxygen (DO) was measured in both 1C and 2C (Pyro Science instrument). pH was determined with a 25+ probe (Crison). The sludge production was determined at the end of the experimental period by collecting the particulate matter settled in the sedimentation unit. Biomass was dried in an oven at 50 ºC for 5 days. Afterwards, the dried biomass was cooled at room temperature in a desiccator and then weighted until reaching a constant weight (given as total solids (TS)).

Scanning electron microscopy (SEM) was used to observe the bacterial colonization in both WE and AE. For visualizing the internal layers of the WE, several

individual carbon fibers were rinsed with deionized water to remove the thick outermost biomass. All the samples were fixed with 5 % (v/v) glutaraldehyde in cacodylate buffer (0.2 M, pH 7.2) and dehydrated through a graded series of ethanol solutions (25, 50, 70, 90 and 100 %; 10 min each stage). Subsequently, the samples were rinsed two times in acetone for 10 minutes and immersed in anhydrous acetone at 4 ºC overnight. Finally, the samples were dried in CO_2 at the critical point and coated with gold. Micrographs were processed using a scanning electron microscope DSM-950 (Zeiss).

The biofilm thickness and mass from the WE was estimated from the thermogravimetric analysis in a Simultaneous Thermal Analyser Mettler Toledo TGA/SDTA851e, under an anoxic atmosphere of N_2. The thermogravimetric curves were taken from 25 ºC to 970 ˚C at an increasing gradient of 10 ˚C min^{-1}. Two samples of the WE were analysed in this equipment: first, a bare sample of electrode of 14.3 mg that served as a control to elucidate the thermal stability of the electrode; second, a sample of WE of 4.1 mg colonized with biofilm, which was submerged in deionized water to remove the non-attached biomass. The biofilm thickness was estimated following the calculations as elsewhere reported (Kramer *et al.*, 2012).

Sampling, DNA Extraction and 16S rDNA Sequencing. Three samples were prepared to determine the composition of their microbial community: 1) biomass from the initial inoculum; 2) biomass from the WE and 3) biomass from the AE. The samples from the electrodes were taken by cutting a piece of the electrodes and then, with tweezers, dipped in three consecutive sterile 0.5 M of EDTA-Na_2 solutions in order to remove loosely attached bacteria. The samples of the inoculum were centrifuged (10,000 g, 2 min) and then washed with the sterile buffer solution twice. Each piece of electrode and pellet was placed into PowerBear tubes, and vortexed for 1 min twice. DNA was extracted with PowerSoil spin columns (MO BIO Laboratories), suspended in 60 µL of sterile MilliQ water and quantified with PicoGreen (Invitrogen). The rDNA sequencing methodology and analysis are described in the Supplementary Information.

Experimental Procedures

A summary of the assays performed and the operating conditions at each one can be seen in Supplementary Table 4-1.

Start-up of the System: Batch Period. Acetate, nitrate and ammonia were added to the medium for the enrichment stage at discontinuous mode to promote nitrifiers and denitrifiers growth. To stimulate the ammonium oxidation in the second

compartment, oxygen was supplied with 2 diffusers that bubbled air from the bottom of this compartment. In order to force the microorganisms to use the electrode situated in 1C as an electron donor, the system was polarized under galvanostatic mode. Current consumption was fixed from -0.044 A m^{-2} to -0.006 A m^{-2} so that this electrode worked as the cathode, and when its potential started to decrease, nitrate was added to the 1C media. After 7 days, the cathode potential, now the WE, was fixed to -700 mV vs Ag/AgCl (potentiostatic mode) in order to favour the nitrate biolectrochemical reduction at high rates as previously observed (Pous *et al.*, 2015). Pulses of nitrate, acetate and ammonia were added again to the media during these 10 days in a discontinuous mode.

Assessment of Different COD/N Ratios in the Influent Under Continuous mode. After the enrichment stage, the system was operated in continuous mode at a hydraulic retention time (HRT) of 4.8 days. It was fed with synthetic wastewater that contained ammonia (NH_4Cl) and acetate ($NaC_2H_4O_2$) in concentrations that depended on the COD/N ratio tested (Table 4-1). Three different COD/N (mass of COD per mass of nitrogen) ratios were tested: 2, 3 and 4. Each assay lasted for at least 3 times the HRT and was repeated twice (the first time, the COD/N was fed at increasing ratios and the second, at decreasing ratios). Samples were taken daily. The WE potential was fixed to -700 mV during all these assays. The influence of DO on the nitrogen removal was tested by bubbling N_2 into the two chambers to flush out the O_2. Additionally, for elucidating the role of the biomass attached to the auxiliary electrode, this electrode was removed from the system and replaced by a titanium mesh structure (abiotic electrode) similar to the current collector used for the AE.

Assessment of Different Working Electrode Potentials for COD/N=4. The effect of the polarization of the electrodes was studied by working under open circuit (OC) conditions and under electrolysis mode. In a first assay, the effect on denitrification by the WE polarization was analysed by maintaining the system at OC for 5 days, feeding the reactor in continuous mode with influent of a COD/N=4 ratio. After that, the working electrode was polarized back to -700 mV. Additionally, the effect of the polarization of electrodes on the nitrification process was studied. For this assay, the reactor media was replaced with one with no organic matter. Ammonium was added in batches to the AE compartment first, with the electrode polarized and second, with the system at OC. Finally, to study the influence of the WE potential on the system's performance, the system was operated again in continuous mode at a ratio of COD/N=4. We varied the WE potentials, testing 5 different values: -470, -590, -700, -980 mV and -1100 mV.

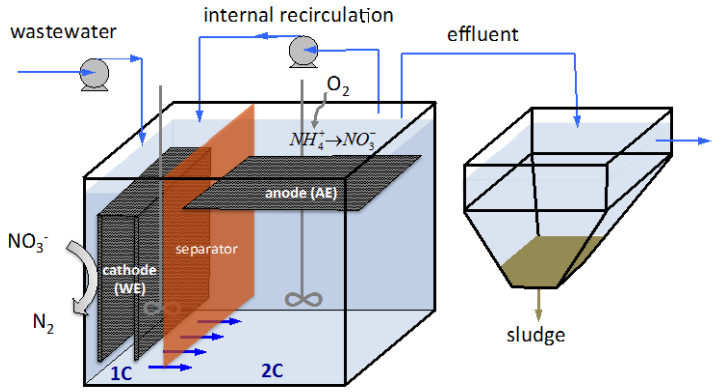

Figure 4-1: Schematic of the reactor design and experimental set-up.

4.4 Results And Discussion

Start-Up Of The System

A schematic of the reactor set-up can be seen in Figure 4-1. For selecting and growing an electroactive population adapted to use the electrode (1C) as electron source, the system was initially operated under galvanostatic conditions. Right after inoculating the reactor, the current was fixed to -0.044 A m^{-2} and the cathode potential gradually decreased until nitrate started to be reduced (see Supplementary Figure 4-1). Due to the drop of the cathode potential to values as low as -1.1 V, the fixed current consumption was decreased to -0.006 A m^{-2} in order to keep the cathode potential over -0.8 V to avoid the production of hydrogen in the electrode, an electron donor competitor. Interestingly, WE potential shifted to higher values when nitrate was spiked in the media, indicating that nitrate was acting as a terminal electron acceptor for microbes. With higher nitrate concentrations in the media, higher cathode potentials were observed. On the contrary, when nitrate was not detected in the media, the potential of the electrode tended to decrease. This means that the set value of current consumption could only be supported by the reduction of other electrons acceptors in the media (such as water) with a reduction potential lower than the one for the microbial nitrate reduction. This behavior of the WE is proof of the bioelectrochemical reduction of nitrate using the electrode as an electron donor. Nitrate was reduced at increasing rates from 41 mg $L^{-1}_{reactor}$ d^{-1} to 533 mg $L^{-1}_{reactor}$ d^{-1} at potentials ranging from -1 to -0.8 V, whereas at potentials from -0.6 V to -0.4 V the rate of removal of nitrate was slower, 22 mg $L^{-1}_{reactor}$ d^{-1}. By day 6.7 the system was switched to potentitostatic mode and the cathode potential (WE) was

fixed to -0.6 V. As a result, the current instantly decreased to -0.05 A m^{-2} until the nitrate was depleted and the current reached a baseline of -0.007 A m^{-2}. Nitrite was detected in the medium as intermediate of nitrate reduction. A rapid return of current consumption was observed whenever nitrate was added to the media. This was an indication of the successful enrichment of electroactive bacteria able to reduce nitrate in the working electrode. After this enrichment stage, nitrate, ammonium and acetate were repeatedly added to the medium for 6 additional days while the system was in potentiostatic mode. Afterwards, the system started to operate under continuous feeding.

Assessment Of Different COD/N Ratios In The Influent Under Continuous Mode

No external aeration was required in our system to remove nitrogen. The DO in the influent and the oxygen that diffused into the reactor media was enough for microbial ammonium oxidation. For all the ratios tested, the nitrification was over 86 % (Table 4-1). It was remarkable that DO levels <1 mg L^{-1} could support the nitrifier's activity, especially at the ratio of COD/N=4, at which DO reached values as low as 0.3 mg L^{-1}. Under this microaerobic environment the nitrification process did not need an external oxygen supply like in classical aerobic activated sludge systems that operate at DO over 2 mg L^{-1}. No nitrite, a nitrification intermediate that is commonly present in low oxygen environments (Ciudad *et al.*, 2006), was detected at any COD/N ratio assayed. This indicates that both nitritation and nitratation reactions were perfectly coupled so that the whole nitrification process was completely accomplished. As the COD/N ratio was increased, the nitrification efficiency decreased (Figure 4-2). We hypothesize this led to the proliferation of heterotrophic microorganism, which have been reported to inhibit the activity of nitrifiers (Hanaki *et al.*, 1990). The fact that the oxidation of ammonium had a similar trend to the DO in the media indicates that oxygen was serving as the electron acceptor for nitrification. When nitrogen was bubbled into the system, the ammonium in the effluent increased suggesting that in the absence of oxygen the nitrification was inhibited (Supplementary Figure 4-2). Since the influent inlet was located in 1C, most of the 1C DO was consumed by aerobic heterotrophs oxidizing the organic matter. This caused a difference in the DO levels in both chambers (Table 4-1). To study the role of the biomass attached to the AE electrode, the electrode was temporally replaced by an electrode of titanium mesh (abiotic AE). The nitrification efficiency started to decrease (Supplementary Figure 4-3) and the DO levels increased in the reactor, which suggests that the oxygen was no longer being utilized by microorganisms. These results confirmed to us that most of the nitrifying activity

was performed by the biomass of the AE. Denitrification was significantly improved when the organic matter in the influent was increased to a ratio of 4, achieving an efficiency of 93 %. Probably the lower levels of DO in the first chamber allowed a better reduction of nitrate because oxygen could not inhibit this reaction, and the concentration of this electron acceptor competitor was lower. This increase in the denitrification efficiency could also be related to the higher availability of electron donor (organic matter).

Since denitrification was performed at COD/N=2 under DO levels from 0.9 to 1.5 mg-O_2 L^{-1}, denitrifying microorganisms may be aerotolerant. However, these denitrifiers could be located at the most internal layers of the biofilm attached to the WE and therefore be exposed to lower oxygen concentrations than measured in the bulk media. No significant changes were found between the ratio COD/N=2 and COD/N=3 in terms of % denitrification, % of nitrification and % of total nitrogen removal (see Supplementary Table 4-2 for the statistical analysis). However, when the COD/N ratio was increased to 4 by enhancing the COD concentration in the influent, the denitrification and the total nitrogen removal were significantly enhanced. Under this condition, the nitrogen removal achieved was of 81 %, corresponding to a rate of 18.7 g N m^{-3} TCC·d^{-1}. This value is slightly lower than others reported in similar studies for removing nitrogen in MET (Virdis *et al.*, 2010) but those required external aeration to oxidize the ammonium. In contrast, it is similar to the rates achieved by Sayess *et al*, that accomplished the nitrification with a rotating cathode that allowed the oxygen diffuse into the media (Sayess *et al.*, 2013) and used higher organic matter concentrations in the influent. In our study, with a COD in the influent as low as 80 mg L^{-1}, 62 % of the nitrogen could be removed. In contrast, when the system was operated with no electrodes in the chambers and with a ratio COD/N =4 in the influent, only 12 % of the total nitrogen was eliminated. In this case, nitrification efficiency accounted for 69 % and denitrification for 17 %. The main limiting step was found to be the reduction of nitrate, as this compound accumulated in the media. Nitrite was detected at trace levels (< 1.1 mg NO_2-N L^{-1}) indicating that nitrification was not completely performed, and the presence of the electrodes is probably stimulating the activity of nitrite oxidizing bacteria (NOB). This showed that the presence of the electrodes was necessary for reducing nitrate under the conditions tested and the absence of them resulted in a system with a very low capacity for removing nitrogen, but still able to oxidize some ammonia to nitrate.

Table 4-1: Operating conditions of the reactor and results of the system performance at the different COD/N ratios tested.

Parameter	COD/N ratio		
Ratio COD/N (mass)	2	3	4
COD influent (mg L^{-1})	80	120	161
N influent (mg L^{-1})	37	37	37
Organic load (g acetate m^{-3} d^{-1})	17.9	26.9	35.8
N load (g-N m^{-3} $_{reactor}$ d^{-1})	8.1	8.1	8.1
Flow rate (L d^{-1})	5.5	5.5	5.5
HRT (d)	4.6	4.6	4.6
COD effluent (mg L^{-1})	34±21	38±12	39±13
N effluent (mg L^{-1})	14±4	13±2	7±2
NH_4^+-N effluent (mg L^{-1})	4±2	5±4	5±3
NO_3^- -N effluent (mg L^{-1})	11±3	9±5	2±1
NO_2^- -N effluent (mg L^{-1})	b.d.l	b.d.l	b.d.l
Nitrification (%)	90±4	88±11	87±8
Denitrification (%)	68±9	75±13	93±4
N removal (%)	62±11	65±5	81±5
COD removal (%)	58±33	68±21	76±25
N removal rate (g N m^{-3}-TCC d^{-1})	14.3±2.5	15.0±1.1	18.7±1.1
COD removal rate (g COD $m^{-3}_{reactor}$ d^{-1})	9.8±5.4	17.1±5.3	25.4±8.4
I consumed (A m^{-2})	-0.10±0.07	-0.29±0.02	-0.39±0.19
DO on 1st chamber (mg L^{-1})	0.9-1.5	0.2-0.5	0.1-0.4
DO on 2nd chamber (mg L^{-1})	1-4	0.6-1.4	0.3-0.8
*** b.d.l: below detection limit**			

When working at electrolysis mode, no significant changes in the COD of the effluent were observed among the three ratios tested, although the removal slightly increased as the COD load did. This could indicate a limitation of substrate for the heterotrophic microorganisms. Regarding the current density consumption in the WE, it was due to two simultaneously competing reactions (the reduction of oxygen and nitrate) and therefore the coulombic efficiency cannot be calculated without knowing the current consumption term due to the oxygen reduction. Our results suggest that the bioelectrochemical electron utilization for reducing nitrate was favored as the organic matter in the influent was increased. Both the current density consumption and the total N removal tended to increase as the COD/N ratio increased. In contrast, the DO in 1C decreased probably due to its consumption by heterotrophs. This enhancement on the utilization of the cathode as electron donor

as the organic matter in the influent increased is in concordance with previous studies which observed that the consumption of oxygen by heterotrophs and nitrifiers benefits the reduction of nitrate with the cathode because the electron acceptor competition in the media is reduced (Yan *et al.*, 2012a).

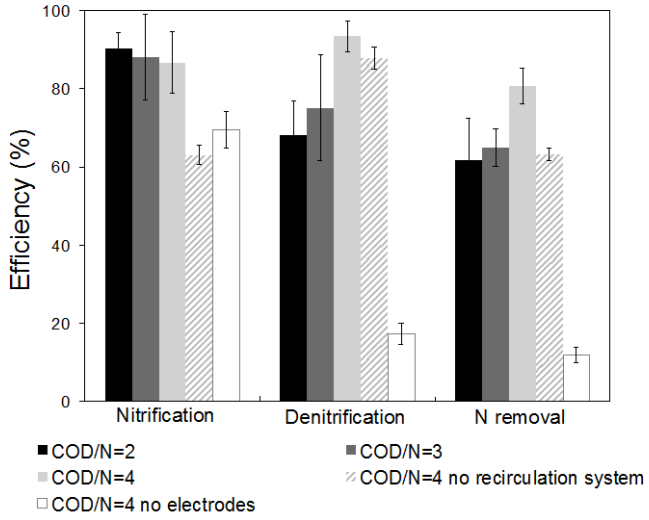

Figure 4-2: Performance of the system in terms of nitrification, denitrification and total nitrogen removal at the different COD/N ratios and scenarios tested.

Operation Of The System Without Internal Recirculation At COD/N=4

Next, the configuration of the reactor was switched to test the reactor's ability to remove nitrogen in the absence of internal recirculation. The circulation in classical reactors moves the nitrate-rich contents of an aerated tank to the anoxic chamber that contains higher levels organic matter (the electron donor). In our case, the electron source was the WE and thus no organic matter was required for denitrifying. Figure 4-2 shows that, in the new configuration without internal recirculation, the nitrification process was significantly inhibited, probably due to the presence of organic matter in the new first chamber (2C in the other configuration). The denitrification process could be almost completely performed, although we should keep in mind that the denitrification rate was lower since *ca*. 40 % of the nitrogen fed remained in the form of ammonium. Overall, this new configuration presented the lowest nitrogen removal among all the bioelectrochemical conditions tested due to the limitations in the nitrification step. Nevertheless, at a ratio of

COD/N=4 and without any external aeration or internal recirculation, this system was able to remove a 63 % of N.

Effect Of The Electrodes Polarization Of The Nitrogen Removal Process

As a control for analyzing the effect of the working electrode potential on the nitrate reduction, the reactor was operated under open circuit conditions with a ratio of COD/N= 4 (Figure 4-3.A). During this period, nitrate accumulated in the effluent. When the WE electrode was repolarized, an instant reduction of nitrate was observed. The nitrate drop was linear (r^2=0.972) within the first 9 hours, and during this time the system was able to reach a constant bioelectrochemical nitrate reduction rate of 48 g NO_3-N m^{-3}-TCC d^{-1}. After that period, the rate gradually decreased until the nitrate concentrations reached values of ca. 10 mg-NO_3 L^{-1}. This suggests that the electroactive bacteria did not lose its electron transfer ability under this period of cathodic electron starvation. Indeed, the electroactive biomass was able to rapidly uptake electrons again from the cathode once this electrode was repolarized. This assay confirmed to us the role of the WE not just as a simple carrier for biomass retention but also as an electron source for denitrifying microorganisms. The effect of the electrodes polarization on the nitrification process was also analyzed (see Supplementary Figure 4-4). The ammonium removal rate was not affected by the electrodes polarization. Higher trace levels of nitrite were measured when the system was operating under OC conditions when compared to the electrolysis mode. Nitrite accumulation was accompanied by a lower nitrate conversion during the first hours. This means that the nitrification process was being performed incompletely during the first hours at OC. Under the conditions tested, the potential of the AE varied between 1.1 to 1.4 V vs Ag/AgCl. The formation of oxygen from water oxidation may have been occurring and thus ammonium-oxidizing microorganisms could have benefited from it. Since the specific affinity of ammonium oxidizing bacteria (AOB) for oxygen is higher than for nitrite-oxidizing bacteria (NOB) (Jones, 2013), under a low oxygen scenario, this electron acceptor is mostly used by AOB. We hypothesize that the production of oxygen at the AE interface could have been supplying oxygen for NOB. In this case, the nitrification process would have been partially assisted by the electrodes.

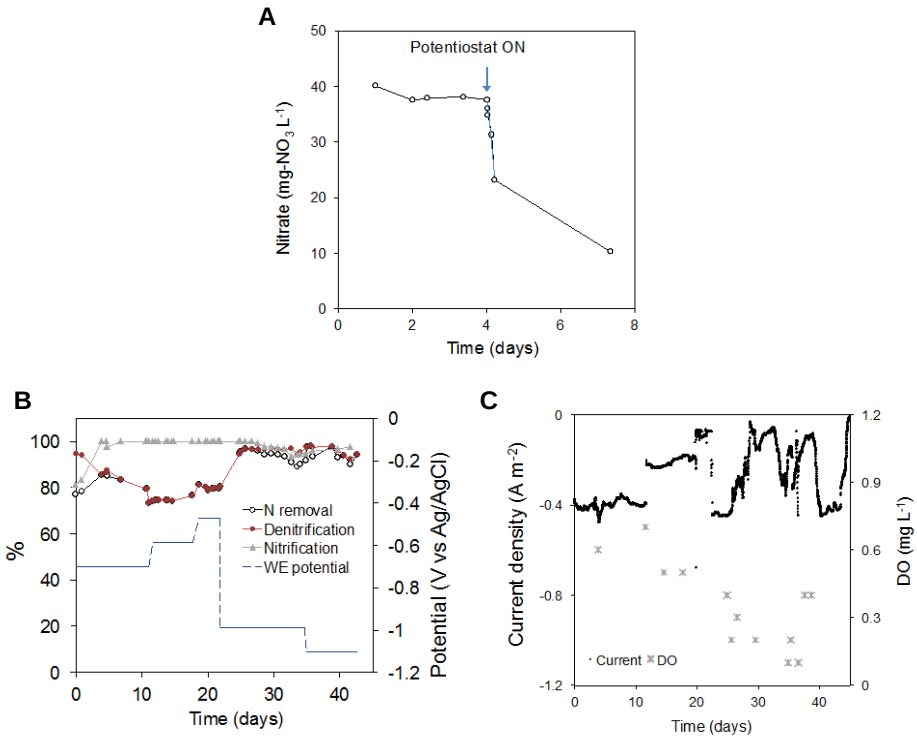

Figure 4-3: Effect of the polarization of the WE on the nitrate reduction. B. Performance of the system in terms of nitrification, denitrification and total nitrogen removal at several fixed WE potentials (all the potentials are reported vs SHE). C. Current density consumed at the different WE potentials and the dissolved oxygen measured in the WE chamber (1C). All the assays were performed at a COD/N ratio of 4.

Finally, in continuous mode (influent with COD/N=4), the effect of the WE potential on the TN removal was tested (Figure 4-3.B). Both nitrification and denitrification were affected by altering the polarization of the electrodes. The highest denitrification rates were achieved when the electrode had a potential of -980 and -1100 mV, reaching values of 96 ± 0.8 % and 98 ± 0.3 % respectively. As a general trend, the highest denitrification efficiencies were achieved at the lowest WE potentials. From the potential of -980 mV to lower values, the nitrification process was slightly inhibited, probably because of the oxygen available was being electrochemically reduced at the electrode faster than it diffused into the media. Actually, both the DO and the current consumption decreased as the WE potential decreased (Figure 4-3.C) whereas the nitrate reduction was barely affected. The nitrification step limited the system's capacity to remove nitrogen when the system was operated at the lowest WE potentials tested. On the contrary, at higher

potentials, the denitrification was the limiting step in the system's performance. In general, decreasing the WE potential favored nitrogen removal because the denitrification process was more sensitive to changes in this parameter than nitrification.

Sludge Production

After all the experimental periods, the sludge from the settling tank of the reactor was collected. The wet sludge had a volume of 0.5 L, and, after being dried, the dried matter weighted a total of 10 g-TS (picture shown at Supplementary Figure 4-5). The COD consumption was estimated considering the total COD added to the reactor, the average COD removal efficiencies for each condition tested and the length of the experiment. With this data we calculated the dry weight produced from all the organic matter consumed, which was of 0.06 kg-TSS kg^{-1} $COD_{removed}$. The sludge production in our system was more than 3-fold less the sludge produced in a similar configuration of activated sludge treating settled wastewater mixed with synthetic wastewater (low TSS content), which is reported to be around 0.2 kg-TSS kg^{-1} $COD_{removed}$ (Ginestet, 2007). This notable difference in the sludge production between the systems could be due to several factors. First, the sludge produced in our reactor comes from the growth of aerobic heterotrophs and from other microorganisms such as anaerobes and electrogens that have lower growth yields (Sokatch, 2014). Second, the low substrate load (organic matter, oxygen and nitrogen) in our system, might have oriented the metabolism of the cells towards a state in which most of the energy from the oxidative reactions is dedicated to cell maintenance. In addition, the presence of the electrodes played a role as electron donor or acceptor but also as a carrier facilitating high concentrations of biomass in the reactor and thus, a high sludge retention time. These two factors also contribute to the establishment of a maintenance metabolism for microorganisms in a system operating under substrate-limiting conditions in a continuous culture. Actually, forcing the biomass to a metabolism primarily for maintenance in wastewater treatment reactors is a known strategy used for reducing the biomass production in activated sludge systems (Wei *et al.*, 2003).

Biofilm Characterization And Microbial Community Analysis

The micrographs of the working electrode showed a thick biofilm composed of microorganisms with very different morphologies (Figure 4-4.A). The outermost layer appeared to be colonized by a dense network of filamentous bacteria (Figure 4-4.A.a and Figure 4-4.A.b). The images of the individual carbon fibers of the WE

(Figure 4-4.A.c and Figure 4-4.A.d) showed the inner most layers of the biofilm, where the morphology was predominantly bacilli. These microorganisms seemed to be interconnected by a matrix of polysaccharides. Information regarding the quantity of the biofilm colonizing the electrode was obtained by thermogravimetric (TG) analysis of a sample of the WE. According to the thermal stability of the main components, the electrode with biofilm can be separated into adsorbed water, organic matter, elemental carbon of the electrode, and mineral salts. The TG profile of the electrode with biofilm showed several weight loss processes as the temperature increased (Supplementary Figure 4-6). The first (until 150 ºC) can be attributed to water loss. Further temperature increase provoked the oxidation of the organic matter with the release of CO_2, H_2O and nitrogen oxides. Finally, when the temperature was high enough, the carbon of the electrode burned via combustion with the release of CO_2, leaving a residue of ash. All these three processes appeared as weight losses in the TG curve and the % of the dry weight (dw) of the biofilm colonizing the electrode would be the sum of the weight loss associated with the oxidation of the organic matter plus a fraction of the weight of the ashes left at high temperatures (mineral phase). The comparative examination of the TG curves of the bare electrode and the electrode with biofilm helped to better identify the weight loss associated with the combustion of the electrode itself. The comparison of the two TG curves also told us that the biofilm was the only source of the ashes. The density of biofilm on the working electrode was of 152 g-dw $m^{-2}_{electrode}$, and the estimated thickness of it was approximately 148 μm. This thickness is significantly higher than those reported in other studies about denitrifying biocathodes (Virdis *et al.*, 2011). Due to this thick layer of biomass, the DO in the media did not probably completely penetrate the biofilm, as Van Loosdrecht and Jetten reported (Van Loosdrecht and Jetten, 1998). Thus, a variety of microbial communities might have colonized the electrode at different depths of the biofilm as a function of the distance from the electrode, and the oxygen, nitrate and ammonium concentrations. Virdis *et al* showed this stratification of a nitrifying and denitrifying microorganisms over a biocathode surface with a biofilm 15-fold thinner than the one in this study (Virdis *et al.*, 2011). In fact, SEM micrographs revealed a bacilli morphology only at the innermost zone of the electrode. The thick biomass layer located the outermost zones of the biofilm, with filamentous microorganisms (Figure 4-4.A (picture a and b)), were probably using the working electrode as a simple carrier rather than as an electron donor.

In order to gain more information about the biofilm that colonized the electrodes, we sequenced the 16S rDNA of the different communities present in the

different environments of the reactor. Several differences were revealed between the microbial composition of the sludge used as the initial inoculum and selected communities in the reactor. The more pronounced changes were found at the bacterial genera level (Figure 4-4.B), which allowed us to go deeper into the metabolic activities in the reactor. Starting with the initial inoculum, *Thauera* spp., *Alishewanella* spp., *Rheinheimera* spp., *Ensifer* spp., *Methylophilus* spp., *Rhodobacter* spp. and *Fusibacter* spp. virtually disappear from selected communities in the reactor. Interestingly, some bacteria that did not have a significant presence in the initial sludge were selected for in the reactor. Among them, *Prosthecobacter* comes to dominate the community in the anoxic chamber with 11.45 %. This genus is characterized by prosthecae, cell wall structures tuboliformes. They present aerobic and oligotrophic metabolism (Hedlund *et al.*, 1996) and they have been found in sludge from the treatment of wastewater.

Our results suggest that oxygen and nitrogen species influenced the selection of the microbial community. Aerobic microorganisms were also enriched in the reactor, of which many are able to reduce nitrates to molecular nitrogen, like *Aromatoleum*, *Azohydromonas* and *Thermomonas* (Mergaert *et al.*, 2003; Xie and Yokota, 2005). Other bacterial genera enriched in the reactor that play a role in the nitrogen cycle are *Solibacter*, that reduces nitrate to ammonium (Ward *et al.*, 2009), *Nitrosomonas*, responsible for autotrophically oxidizing ammonium to nitrite, and *Nitrospira*, capable of oxidizing nitrite (Lücker *et al.*, 2010). Interestingly, the *Nitrospira* genus was notably more abundant in the AE electrode, located at 2C, where the highest levels of oxygen were measured. This confirms that most of the nitrification activity was concentrated in the AE chamber. Denitrifying genera such as *Opitutos*, *Methyloversa* (Baytshtok *et al.*, 2009) and *Zoogloea* were enriched in the reactor, the last two being more abundant in the working electrode biofilm. The genus *Zoogloea* is known to be capable of reducing both oxygen and nitrate. They also form gelatinous matrixes and flocs, that could be related to the matrix observed on the micrographs from the working electrode (Shao *et al.*, 2009). Interestingly, although this genus is typically found in sewage samples, some studies have found it in iron sediments or in paddy soils and is reported to play a role in the iron cycle (Coates *et al.*, 1998; Watanabe *et al.*, 2013). The presence of this denitrifying genus in the biofilm of the working electrode could be an indication of this genus´s ability to accept electrons from the cathode. The denitrifying bacteria constituted from 4.5 to more than 7 % of the community, confirming a high activity of nitrate reduction therein. Other differences among communities in the WE and the AE were found in

Chondromyces (from 2 to 0.53 %), *Chthoniobacter* (from 0.68 to 1.44 %) and *Prosthecobacter* (from 18 to 0.2 %).

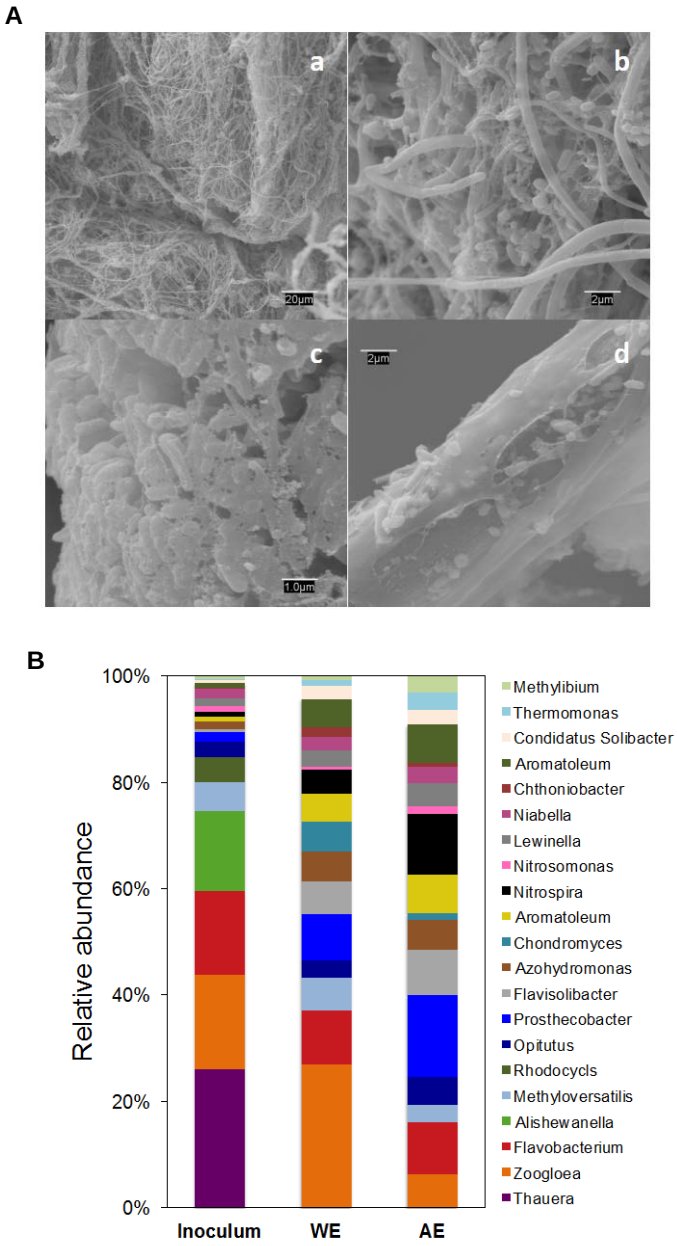

Figure 4-4: A. Micrographs of the biofilm formed on the working electrode surface. B. Microbial community diversity at the genera level in the initial inoculum and in the biomass attached to the working and the counter electrodes.

Regarding the phyla-level changes, after 18 months of operation Firmicutes almost disappeared, going in the initial sludge from 2.6 to 0.2-0.3%, while the Verrucomicrobia (1.4 to 5.5 - 15 %) and Nitrospirae (0.2 to 1.7 - 4.5 %) were enriched in the reactor (Figure 4-5.A). Firmicutes are known for their ability to degrade and ferment complex carbohydrates, which were not in the synthetic sewage. The increase of the other three phyla can be explained by the presence of a higher concentration of nitrates in the media and a decrease of nutrients, when compared to regular wastewater. Meanwhile, the main classes that were enriched in the reactor were Sphingobacteria (in both the WE and AE), Deltaproteobacteria (in the WE), Planctomycea and Nitrospira (both in the AE) (Figure 4-5.B). Although this analysis can provide us with information about the microbial activity in our reactor, it is difficult to place the specific role of the different electrode-attached populations due to the large thickness of the biofilm. Likely, only a reduced section of the biofilm was directly or indirectly interacting with the electrode.

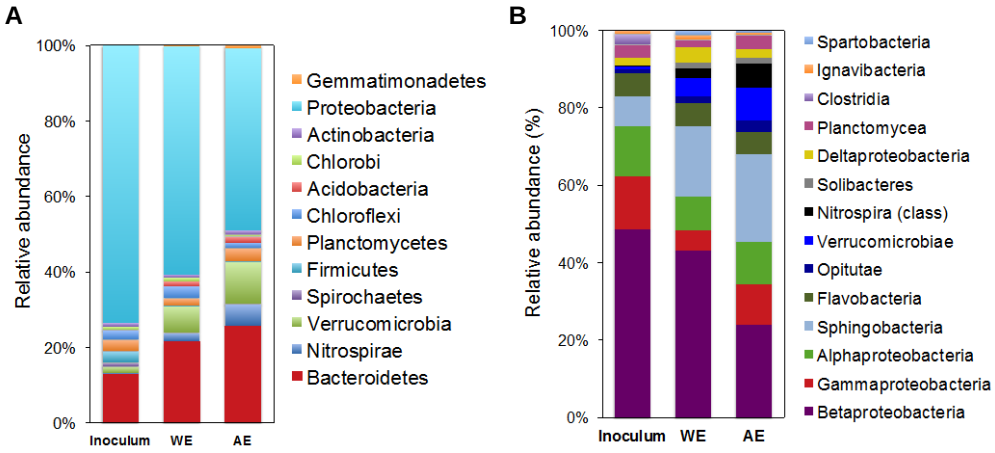

Figure 4-5: Relative abundance of the different microbial communities sorted by phylum (A) and class (B) of the three samples analyzed. WE stands for the working electrode attached biomass and AE stands for the one attached to the auxilar electrode.

4.5 Conclusions

This study demonstrates the viability of reusing or converting a classical activated sludge reactor into a membrane-free MET reactor for removing nitrogen and carbon from low organic matter wastewater. This would allow the reuse of infrastructure already build decades ago and that are currently being replaced by new advance bioreactors for treating the wastewaters. One the main benefits of converting an activated sludge system into a MET system is that investment costs of

this technology are limited to the electrochemical equipment because the energy consumption during the operation is highly reduced due to the elimination or minimization of the need for supplied oxygen. We propose our system as a third treatment of effluents that come from a previous carbon removal step, which is unable to remove nutrients (like an anaerobic digester). The nitrogen removal was feasible due to the presence of a biocathode, which served as electron source for denitrifying microorganisms. In our 2-chamber system with internal recirculation, the nitrification process, not mediated by the electrodes, could be coupled to the electrogenic denifriticacion step. Our reactor was able to remove nitrogen from a low organic matter influent with no external aeration and no membrane separation between the two electrode chambers and all while reducing the sludge production. Further research should work towards optimizing some of the key system parameters such as, the working electrode potential, the recirculating flow, and the design of the electrodes (the area, the material and the position within the chambers). Besides, it is recommended to perform a control on the AE potential in order to avoid the oxidation of chloride ions to chorine gas, which would negatively affect the biomass in the reactor. This study shows that it is possible to adapt METs to already built activated-sludge reactors and integrate them into urban wastewater treatment plants with no extra infrastructure costs beyond electrode installation. The energy demand in this system was from 0.12 to 0.7 kWh $m^{-3}_{wastewater}$ (0.005 kWh $g\text{-}N^{-1}$ to 0.024 kWh $g\text{-}N^{-1}$) and highly varied depending on the system performance (see Supplementary Information). These values are higher than others reported for nitrogen removal technologies (Mulder, 2003) as activated sludge configurations. However, in our system the low sludge production can highly reduce the extra costs associated with its management. The economic viability of this system relies on the reduction of the energy demanded by the potentiostat or power source used in the MET, which implies minimizing the internal resistance and the electrodes overpotential (optimization of its area and material).

Acknowledgements

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4.6 Supplementary Information

Supplementary Table 4-1: List of assays performed with the system and the operating conditions at each one.

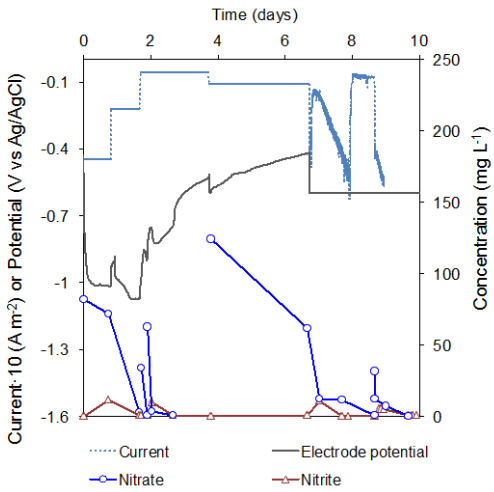
Time (days)	Electrochemical condition	Operation mode	Ratio
0-7	Galvanostatic	Batch mode	-
7-17	Potentiostatic (-500 vs SHE)	Batch mode	-
18-83	Potentiostatic (-500 vs SHE)	Continuous mode	COD/N=2
84-108	Potentiostatic (-500 vs SHE)	Continuous mode	COD/N=3
109-234	Potentiostatic	Continuous mode - Study at -500 vs SHE - Study at effect of cathode polarization and different potentials - Study of factors affecting the system: role of AE, effect of O_2	COD/N=4
235-261	Potentiostatic (-500 vs SHE)	Continuous mode	COD/N=3
262-327	Potentiostatic (-500 vs SHE)	Continuous mode	COD/N=2
328-361	Potentiostatic (-500 vs SHE)	System without internal recirculation	COD/N=4

rDNA Sequencing Methodology and Analysis

A total of 3 ng of DNA were amplified with primers 515F-CS1 (ACACTGACGACATGGTTCTACA*GTGCCAGCMGCCGCGGTAA*) and 806R-CS2 (TACGGTAGCAGAGACTTGGTCT*GGACTACHVGGGTWTCTAAT*; underlined the sequencing primers, in italics the 16S rDNA-specific primers). The polymerase used was Q5 Hot Start High-Fidelity (New England Biolabs) and the PCR conditions were: initial denaturation at 98°C for 30'' followed by 30 cycles of 98°C x 10'', 60°C x 20'' and 72°C x 20'', and a final elongation step of 72°C for 2'. A 1/100 dilution of PCR products were then re-amplified (15 cycles) with Illumina´s primers AATGATACGGCGACCACCGAGATCTACACTGACGACATGGTTCTACA and CAAGCAGAAGACGGCATACGAGAT-[BC]-TACGGTAGCAGAGACTTGGTCT, where BC represent the 6 nucleotides long barcode. Positive reactions were excised out of the gel in order to get rid of any possible primer-dimers and undesired products. Finally, products were run on a Bioanalyzer (Agilent) to estimate the

concentration of each simple within the region of interest and the successful generation of equimolar pools was confirmed by qPCR. Sequencing was performed in a MiSeq equipment using the 2x250 bp format and following Illumina's protocol.

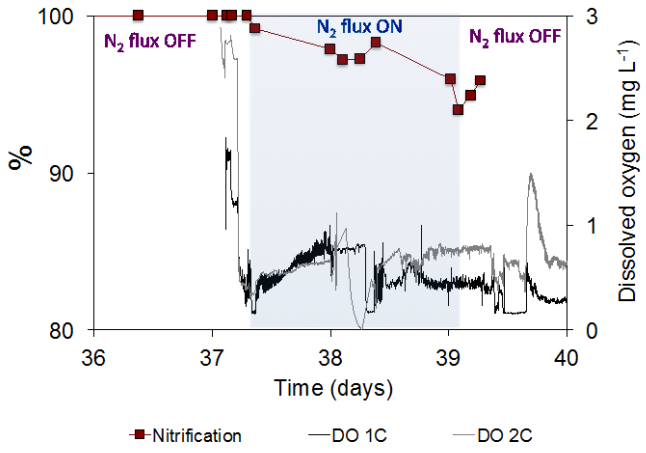
A total of around 1.000.000 sequence reads were obtained and analyzed with the QIIME 1.7 pipeline (Caporaso *et al.*, 2010) with few stiches along the way. Briefly, complementary reads were merged using fastq-join (Aronesty, 2011). Subsequently, our quality filtering strategy removed complemented sequences that had one of the following characteristics: (i) deviated more than 10 bp from the expected length (292); (ii) contained primers with more than 1 mismatch or; (iii) contained nucleotides with Phred score <20. Filtered seqs were organized in OTUs by *de novo* picking using Usearch (Edgar, 2010) and one representative sequence per OTU was chosen. Taxonomy was assigned using the GreenGenes database (DeSantis *et al.*, 2006) version 10_12 at the 97 % identity rate. Furthermore, sequences were aligned and a tree generated using FastTree 2.1.3 (Price *et al.*, 2010). Finally, in order to investigate alpha diversity and the network formed by communities members with QIIME, OTUs containing less than 0.005% of the total sample reads were removed according to Bokulich (Bokulich *et al.*, 2013). The resulting network was analyzed and visualized using Cytoscape (Shannon, 2003).



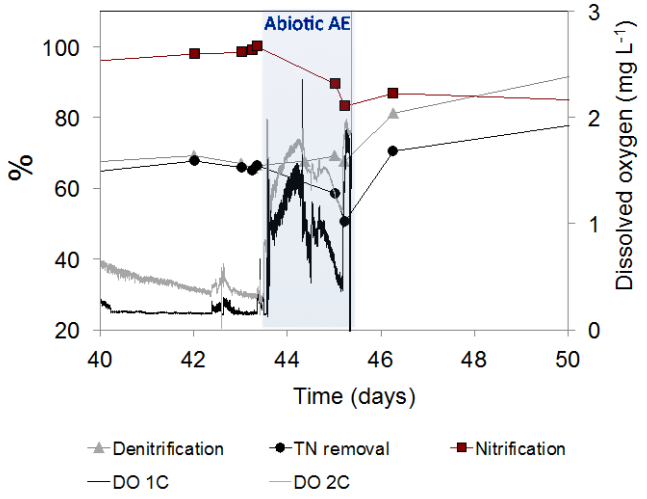
Supplementary Figure 4-1: Current, potential of the WE (vs Ag/AgCl) and nitrogen species concentration during the start-up period at batch mode.

Supplementary Table 4-2: Student's t-test for each pair of variables compared. The compared variables are the ones represented in Figure 4-2. The confidence interval was of 95 % and the significance level of 0.05.

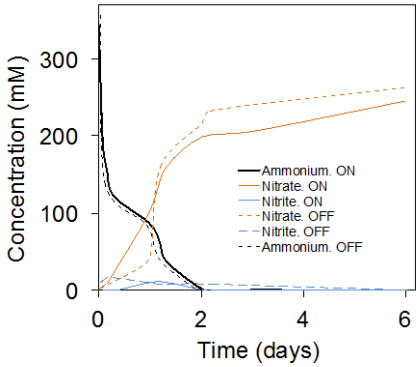
Compared variables	Student's t	Degrees of freedom	Probability<t	Significant difference
% Nitrification for COD/N 2 and 3	-2.37	11	0.980	NO
% Nitrification for COD/N 3 and 4	7.854	11	1.38E-05	YES
% Nitrification for COD/N 2 and 4	4.367	11	7.03E-04	YES
% Nitrification for COD/N 4 and COD/N 4 without electrodes	6.356	8	7.12E-14	YES
% Nitrification for COD/N 4 and COD/N=4 without recirculation	12.78	8	6.62E-7	YES
% Denitrification for COD/N 2 and 3	0.744	11	0.76365	NO
% Denitrification for COD/N 3 and 4	-31.759	11	1.79E-12	YES
% Denitrification for COD/N 2 and 4	-10.533	11	2.20E-07	YES
% Denitrification for COD/N 4 and COD/N 4 without electrodes	63.106	8	9.46E-9	YES
% Denitrification for COD/N 4 and COD/N 4 without recirculation	5.20	8	4.11E-4	YES
% Total N removal for COD/N 2 and 3	-0.324	11	0.37614	NO
% Total N removal for COD/N 3 and 4	-10.935	11	1.502E-07	YES
% Total N removal for COD/N 2 and 4	-5.105	11	1.71E-04	YES
% Total N removal for COD/N 4 and COD/N 4 without electrodes	3.245	8	1.142E-2	YES
% Total N removal for COD/N 4 and COD/N 4 without recirculation	7.949	8	2.29E-5	YES



Supplementary Figure 4-2: Effect of removing the dissolved oxygen in the reactor over the system performance by bubbling N_2.



Supplementary Figure 4-3: Effect of the removal of the auxiliar or counter electrode and replaced by an abiotic one of titanium.



Supplementary Figure 4-4: Effect of the polarization of the electrodes on the nitrification process. The assay was performed at batch mode and with a medium containing ammonium and acetate at a ratio COD/N=4. ON stands for the polarization of the electrodes condition, whereas OFF stands for the open circuit potential condition.

Supplementary Figure 4-5: Dry sludge production during all the operation period.

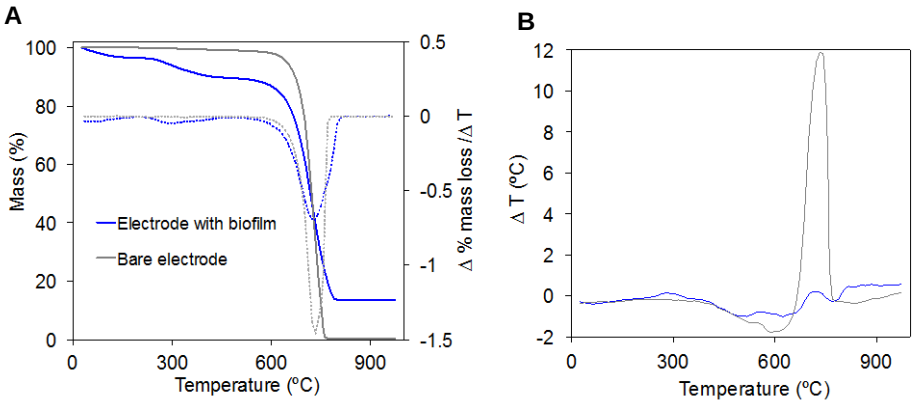
Biomass density estimation on the working electrode

Biomass density estimation on the working electrode

The TG curve for the bare electrode (Supplementary Fig.4-6.A) shows a single weigh loss between 25 and 1000 ºC with an initial temperature near 600 ºC. This process shows in the simulteneous differential thermal analysis (STDA) curve (Supplementary Fig.4-6.B) as a single exothermic peak. The TG curve of the electrode with biofilm shows three weight losses. The first between 25 and 150 ºC, is associated with water loss. The second one, between 200 and 400 ºC, should be attributed to the presence of microbial matter, as it does not show in the TG curve of the bare electrode. Also, the comparative examination of the TG curves of the bare

and used electrode shows that the ashes are entirely originated from the combustion of the biofilm. Thus, the percentage of dry weight of biofilm in an electrode should be the sum of the weight loss between 200 and 400 ºC and the weigh of the ashes. The mass loss attributed to the biofilm was of 1.14 mg for an electrode area of 0.075 m^2. Considering a density of 1.03 g-dry weight cm^{-3}-biofilm and a uniform thickness over the electrode area, the estimated thickness of the biofilm was of 148 μm.

The SDTA curve of the electrode with biofilm shows an exothermic peak between 200 and 400 ºC, as expected for the oxidation of the organic matter. The weight loss at 600 ºC in the TG curve appears in this curve as two small exothermic peaks in place of a single large exothermic peak shown in the STDA curve of the bare electrode. This means that the combustion of the carbon of the electrode with biofilm has a different mechanism that for the bare electrode. The profile of the SDTA curve identify the mechanism of the burning process of this electrode as a heterogeneous -homogeneous combustion (Li *et al.*, 2009). The change of mechanism can probably be caused by the presence of the mineral phase coating the fibers of carbon and decreasing the oxygen diffusion from the atmosphere to the surface of the carbon preventing its direct oxidation.



Supplementary Figure 4-6: A. TG profiles of both the working electrode covered with biofilm (continuous line) and a bare electrode (doted line). The black lines indicate the percentage of mass loss of the samples, and the blue lines their corresponding first derivative. B. Simulteneous differential thermal analysis (SDTA) curves of the two samples.

Energy demand calculations

The energy demand was calculated for the lowest system performance in terms of nitrogen removal (less current consumption due to a lower bioelectrochemical nitrate reduction), and for the highest nitrogen removal condition.

Area electrode = 0.09 m^2
Flow = 4.6 L day^{-1}
Power consumption = $\Delta E(E_{node}\text{-}E_{cathode})\cdot I_{consumed}$

- Energy consumption for COD/N=2 (lowest N removal):

Current consumption was of 0.10 A m^{-2} and the nitrogen removal was of 62 % of an influent with 37 mg-N L^{-1}. The potential of the cell (E_{anode}-$E_{cathode}$) for this condition was of *ca.* 2.5 V. The energy demand for this ratio was of 0.12 kWh $m^{-3}_{wastewater}$ or 0.005 kWh g-N^{-1}.

- Energy consumption for COD/N=4 (highest N removal):

Current consumption was of 0.39 A m^{-2} and the nitrogen removal was of 81 % of an influent with 37 mg-N L^{-1}. The potential of the cell (E_{anode}-$E_{cathode}$) for this condition was of *ca.* 4 V. Therefore, the estimated energy demand for this condition was of 0.7 kWh $m^{-3}_{wastewater}$ or 0.024 kWh g-N^{-1}.

Part II: Merging Microbial Electrochemical Systems With Electrocoagulation Pretreatment for Achieving a Complete Treatment of Brewery Wastewater

This section has been redrafted after:

Sara Tejedor-Sanz[a,b], Juan Manuel Ortiz[b,c] and Abraham Esteve-Núñez[a,b]. 2016. *Merging microbial electrochemical systems with electrocoagulation pretreatment for achieving a complete treatment of brewery wastewater.* Submitted.

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Merging Microbial Electrochemical Systems with Electrocoagulation Pretreatment for Achieving a Complete Treatment of Brewery Wastewater

4.7 Abstract

The limitations of microbial electrochemical technologies (METs) for full-scale wastewater treatment suggest the need for supporting these systems with a complementary technology. In this study we propose the integration of two different electrochemical techniques to fully treat brewery effluents: an electrocoagulation (EC) and a microbial electrochemical fluidized bed reactor (ME-FBR). The EC pretreatment efficiently removed most of the suspended matter that contained the insoluble chemical oxygen demand (COD) and most of the nutrients. We investigated the influence of current density and the reaction time on the EC performance. The effluent of the EC was continuously fed into a ME-FBR with a fluidized and polarized anode colonized with electroactive biofilm. This second step oxidized the organic matter using the fluidized bed as terminal electron acceptor. With this novel combination of techniques, it was possible to remove a 93 % of nitrogen, 98 % of phosphorous, 93 % of the total suspended solids, and > 88 % of the COD of a brewery wastewater.

4.8 Introduction

Microbial electrochemical technologies represent a promising field based on the effective redox coupling between microbial metabolism and electrically conductive materials (Du *et al.*, 2007). In the wastewater treatment field these novel systems can potentially represent an alternative to classical technologies due to its energy-saving benefits (Sleutels *et al.*, 2012).

Although urban wastewater (Brown *et al.*, 2015; Min and Logan, 2004) has been the most common biodegradable fuel tested in METs, industrial organic matter sources such as food industry residues have been extensively tested in the last decade (Cercado-Quezada *et al.*, 2010; Çetinkaya *et al.*, 2015; Kelly and He, 2014a). Specifically, the bioelectrochemical treatment of brewery wastewater has received much attention (Dong *et al.*, 2015; Feng *et al.*, 2008). In this wastewater the organic matter is classically treated by aerobic methods or by anaerobic digestion in UASB or fluidized bed reactors, whereas nutrients (N and P) are eliminated in aeration tanks or by physic-chemical processes (Simate *et al.*, 2011). Nutrients

removal in bioelectrochemical systems is one of the main challenges that the field faces. Although some studies have reported simultaneous organic matter and nutrients removal from wastewaters in METs (Virdis *et al.*, 2010; Zhang *et al.*, 2011), there are no reports describing scalable prototypes able to remove both N and P removal. The degradation of organic matter in brewery effluents in bioelectrochemical systems has been extensively studied, however, none of these reports have proposed a method for removing the nutrients (Dong *et al.*, 2015; Feng *et al.*, 2008; Köroğlu *et al.*, 2014). Furthermore, the main bottlenecks for scaling-up METs are the problems related to using biofilm-based electrodes: the mass transfer limitation and the low active surface area of the electrode (Scott and Yu, 2015). To deal with them, METs can benefit from a novel concept where the classical static electrode is replaced by a mobile-like electrode which can act as a 3D electrode carrier for biomass growth. On top of that, it assures a proper mixing inside the reactor (Heijnen *et al.*, 1989) and a high mass transport. In that sense, some mobile bioanodes, either constructed of stirred conductive granules (Liu *et al.*, 2014b) or made of capacitive conductive granules (Deeke *et al.*, 2015) have been reported to accept electrons from mixed populations.

In this context, we propose a complete process based on the integration of two electrochemical techniques for treating a brewery wastewater and that could be extrapolated to other kind of industry effluents. First, an electrocoagulation (EC) step where suspended solids and bound nutrients are removed. Second, we have merged a classical fluidized bioreactor concept with METs resulting in Microbial Electrochemical-Fluidized Bed Reactor (ME-FBR). The aim of this work is to demonstrate that the integration of these two technologies is a suitable strategy for the complete treatment of industrial brewery wastewater.

4.9 Materials And Methods

Wastewater Description and Analysis. All wastewater samples used for the experiments were collected on the same day from the brewery plant Mahou-San Miguel in Alovera, Guadalajara, and stored at 4ºC until used. Wastewater samples were taken from the homogenization tank that harvests different effluents of the brewery production line.

Analytical Methods. Wastewater samples were analyzed according to the Standard Methods for the Examination of Water and Wastewater (Eaton and Franson, 2005). Samples were frozen at -20ºC prior to their analysis. COD, total N and total P were measured with a commercial kit in a Spectroquant TR420 and a

Spectroquant Pharo 100 from Merck. Total organic carbon (TOC) (soluble fraction) analyses were performed in an Organic Carbon Analyzer TOC-V CSH from Shimadzu. Total suspended solids (TSS) were determined by vacuum filtration using AP40 90 mm filters from Millipore. The conductivity was measured with a conductimeter MM 41 and the pH with pHmeter pH 25, both from Crison. The turbidity was determined with a TubiDirect (Lovibond), and the color with a PCcheckit (Lovibond). Alkalinity tests were performed by titration system model 809 from Metrohm. Ammonium and nitrate were measured in a Metrohm Advanced Compact IC model 861 with two channels.

Electrocoagulation Procedure. The electrocoagulation cell consisted of a cylindrical vessel with a capacity of 1.5 L, in which four electrodes, two of aluminum (anode) and two graphite plates (cathode), were immersed. The electrode size was 13 x 9 cm, the thickness of the aluminum plates was 0.1 cm, while for graphite plates was 0.2 cm. At the top, each electrode had a small rectangular contact (6.5 x 2 x 0.1 cm) used to connect wires. The separation between electrodes was 1 cm, with each aluminum electrode facing a graphite electrode. In order to keep that spacing constant, the electrodes were attached to two nylon rods at their uppermost parts. EC experiments were performed using a power supply Elektro-Automatik PS 2016-100 (0→16V,0→10A) and a Fluke 177 True RMS multimeter was used for measuring the electric current and voltage applied. The EC cell was constantly stirred at 900 rpm. All experiments were performed at room temperature (23-28 ºC). Two sets of experiments were performed in the EC. First, the reaction time (RT) of the wastewater in the cell was maintained constant (15 min) and the current density (*j*) was varied (2.6, 5 and 10 mA cm^{-2}). Second, *j* was fixed to 5 mA cm^{-2} and the RT was changed (5, 10 and 15 min). The last step of the process was filtration using filter paper. Raw wastewater was renewed in the cell for each test. The treatment capacity (TC) for each assay was calculated by dividing the volume of wastewater treated (1.5 L) by the RT and the anode area.

Microbial Electrochemical- Fluidized Bed Reactor (ME-FBR). The description of the ME-FBR used can be found in Chapter 3. An Ag/AgCl 3 M KCl electrode (HANNA) was employed as a reference electrode. The ME-FBR was operated as a three-electrode electrochemical cell and the fluidized bed worked as the anode by polarizing it to 0.2 V (all potentials are reported versus Ag/AgCl electrode). The potentiostat used was a NEV3 Nanoelectra. The reactor was inoculated with sludge from a wastewater treatment plant and was previously operated during 5 months first at batch mode and second, at continuous mode, with brewery wastewater as influent without EC pretreatment. Therefore, the biomass

was already adapted to the feeding and the fluidized particles were colonized with electroactive biofilm. A peristaltic pump (Watson and Marlow, 205S) was used for continuously feeding the reactor with wastewater from an inlet port placed at the bottom of the column. The effluent outlet port was located at upper part of the reactor. The hydraulic retention time in the ME-FBR was of 2.4 days. Samples were collected dairy and kept at -20 ºC for subsequent analysis.

4.10 Results And Discussion

The analytical results from the brewery wastewater characterization (Table 4-2) showed a complex effluent with a high content of TSS and COD. Ammonium and nitrate were below the detection limits, indicating that all the nitrogen in the wastewater was in the form of suspended and complex matter. Furthermore, phosphorous concentration in the raw wastewater was relatively high (15 mg L^{-1}). We proceed to treat the effluent through two sequential stages: first, in an EC cell and second, in a ME-FBR. Figure 4-6 shows a diagram of the combined process used with the possibility of recovering added-value by-products from the treatment.

Table 4-2: Chemical and physical parameters of the brewery wastewater and the effluents after both EC and ME-FBR treatments.

Parameter	Brewery WW	Effluent from EC	Effluent from ME-FBR
TOC (mg L^{-1})	890±60	840±15	120±30
COD (mg L^{-1})	2900±150	2760±90	360±40
NO_3^- (mg L^{-1})	n.d	4±1	1±1
NH_4^+ (mg L^{-1})	n.d	7±1	n.d
Total N (mg L^{-1})	66±5	9±2	<5
SO_4^- (mg L^{-1})	21	12±1	5±1
Total P (mg L^{-1})	15.1±1	0.6±0.1	0.3±0.2
TSS (mg L^{-1})	625±23	70±23	47±17
Alkalinity (mg L^{-1} $CaCO_3$)	1163	1087	1330
pH	7	8.2	9.1
Conductivity (mS cm^{-2})	2.8	2.6	2.50
Turbidity (UNT)	447	29	6.3
Color (mg L^{-1}Pt-Co)	4180	315	110

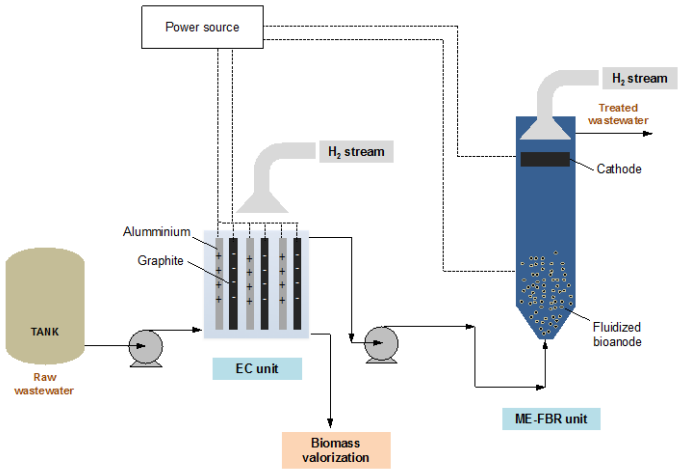

Figure 4-6: Schematic of the process proposed for the treatment of a brewery wastewater as a sustainable methodology with added-value by-products (hydrogen and the biomass from the EC).

Electrocoagulation For Removing Solids And Nutrients.

Electrocoagulation is an electrochemical technique closely related to chemical coagulation. It involves supplying coagulant ions (Al^{3+}, Fe^{3+}) by the application of an electric current to a sacrificial anode (made of aluminum or iron) placed into a processing tank (Cañizares *et al.*, 2005; Mollah *et al.*, 2004; Tian *et al.*, 2016). Some of the advantages of this technology are the low amount of chemicals needed, the low costs of operation and the fact that the salinity of the effluent is not increased (Mollah *et al.*, 2001).

When our raw brewery wastewater was treated by EC, the removal of the colloidal matter in it was significant in all tests performed. Figure 4-7.A shows the experiments for constant current density at different TC. As it is observed, for the lowest TC tested, 0.17 m^3 m^{-2} h^{-1} (RT=15 min), a 96 % of P and 98 % of N was removed from the wastewater. Meanwhile, the COD content decreased by 20 % and the TSS by 89 %. The performance was significantly affected by the variation of the TC of the cell. By decreasing this parameter, the operating time of the EC cell with the wastewater was enhanced. Thus, a larger amount of coagulant was electrogenerated and, as a result, a larger amount of suspended particles were destabilized. However, the decrease in the removal of nutrients and COD did not show a proportional relation with the TC. For example, operating the EC cell with a

3-fold increased TC (from 0.17 m^3 m^{-2} h^{-1} to 0.51 m^3 m^{-2}_{anode} h^{-1}) did not produced a proportional reduction on the removal of COD, TSS, N and P. Additionally, when the TC in the cell was reduced by half, (from 0.51 m^3 m^{-2}_{anode} h^{-1} to 0.26 m^3 m^{-2}_{anode} h^{-1}) the removal of P and N, clearly marked, and of the COD, was higher than 2-fold. These effects could be related to the time needed for the aggregates and flocks to be formed in the bulk medium.

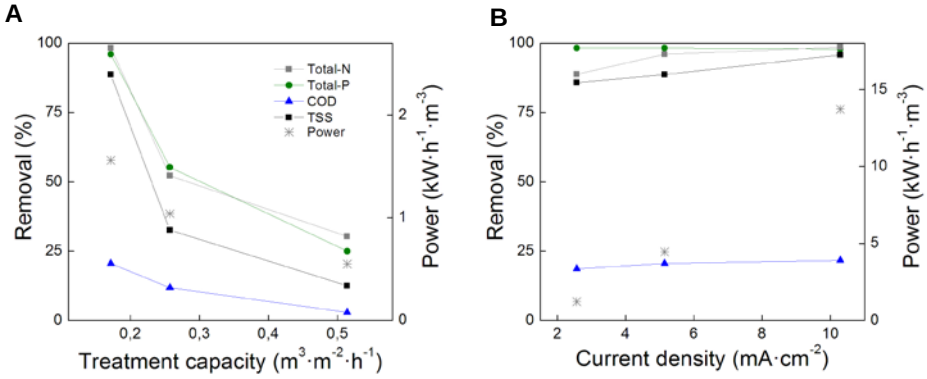

Figure 4-7: Removal of COD, TSS and nutrients, and power consumption of the EC cell at the different tests. A: At different treatment capacities and at a fixed current density of 5 mA cm^{-2}. B: At different current densities and at a constant treatment capacity of 0.17 m^3 m^{-2} h^{-1} (RT=15 min).

The results for the series of assays where *j* was the variable term and the TC remained constant are shown in Figure 4-7.B. By applying higher *j* in the EC cell, the coagulant (i.e. $Al(OH)_3$) was electrochemically generated at higher rates. This could either increase the TC of the cell or decrease the required reactor size. In our EC unit, increasing *j* from 2.6 to 5 mA cm^{-2} did not show any difference in the removal of P whereas the removal of N, COD, and TSS showed a slight enhancement, suggesting that both could be further eliminated by electrogenerating more coagulant. Increasing further *j* to 10 mA cm^{-2} did not significantly affect the removal rate of nutrients and COD, indicating that all the particulate matter capable of being destabilized had been at 5 mA cm^{-2}. Regarding COD removal, we can assume that under non-limiting coagulant (i.e. $Al(OH)_3$) conditions, the remaining COD was due to the soluble fraction contained in the wastewater. Specifically, COD could not be removed further than 21 %, a result that gives us an idea of the proportion of insoluble COD in the brewery wastewater. It is remarkable that an optimal EC performance was able to reach levels of nutrients as low as 0.2 mg L^{-1} of P and 1.2 mg L^{-1} of N. One of the benefits of using EC is that these concentrations can be tuned by varying parameters such as *j* or the TC. Our results revealed that nutrients

removed by the EC treatment were mainly associated to the particulate matter present in the wastewater. In the raw brewery effluent, the initial ratio COD:N:P was of approximately 500:11:3 (mass units), and after the EC treatment, this ratio was decreased to values as low as 500:0.4:0.1. This ratio is below the nutrients requirements reported for the biomass growth in anaerobic digesters (Annachhatre, 1996). However, it was enough for satisfying the anabolic demand of the microbial community in our ME-FBR during the experimental period.

Finally, optimal operation conditions of the EC, in terms of removal performance with minimum energy consumption, were established (j=5 mA cm^{-2}, TC=0.17 m^3 m^{-2} h^{-1}) to generate an effluent ready to be treated in the following step: the ME-FBR. Information regarding the aluminum and energy consumption on the EC cell is available in Table 4-3.

Table 4-3: Analytical results from the different tests in the EC and the estimated power consumption, aluminum consumption and treatment cost (based on a value of electricity cost of 0.1 € kWh^{-1}).

Test	Treatment capacity (m^3 $h^{-1}m^{-2}$)	Current density (mA cm^{-1})	Power (kWh m^{-3})	COD removal (%)	Total P removal (%)	Total N removal (%)	Al consumption (g m^{-3}-treated water)	EC cost* (€ m^{-3})
1	0.51	5.14	0.55	2.93	25.0	30.4	1.24	0.060
2	0.26	5.14	1.04	11.7	55.3	52.2	2.49	0.114
3	0.17	5.14	1.56	20.5	96.0	98.2	3.73	0.17
4	0.17	2.57	1.33	18.6	88.8	98.3	1.86	0.508
5	0.17	10.3	13.71	21.7	98.6	98.0	7.46	0.053

Microbial Electrochemical-Fluidized Bed Reactor For Removing Soluble COD

The COD in the influent of the ME-FBR had mostly soluble matter as minor fermentation compounds (sugars), soluble protein matter and organic acids. Thus, fermentation processes, conversions of complex organics into volatile fatty acids (VFAs) by acidogens, acetogenesis, microbial electrogenesis (current harvesting), and methanogenesis were simultaneously occurring in the ME-FBR. This configuration operated at an organic loading rate of 1.15 kg-COD m^{-3}·d^{-1} and efficiently removed 87 % of the total COD. Moreover, more than 50% of the TSS that remained in the wastewater after the electrocoagulation step was further

removed, reducing the turbidity and color of the effluent. Nitrogen and phosphorous were used by microorganisms for biomass synthesis since its concentration decreased further after the ME-FBR treatment, remaining in the final effluent at trace levels (Figure 4-8). Interestingly, sulphate was reduced in the ME-FBR by a 63 %, revealing the presence of sulphate reducing bacteria (SRB). These bacteria compete with electrogens for VFAs and even for other sources of electrons in the wastewater such as ethanol, aromatic compounds or the hydrogen produced either at the cathode or by acetogenic bacteria (Oremland and Taylor, 1978). However, if we estimate the theoretical COD required to reduce all the sulphate in the wastewater, it accounts for less than 1% of the organic matter available in the ME-FBR influent. The ME-FBR operation produced an alkalinization of the effluent that eventually reached a pH of 9. The consumption of VFAs in the medium could have been responsible of the alkalinization of the effluent.

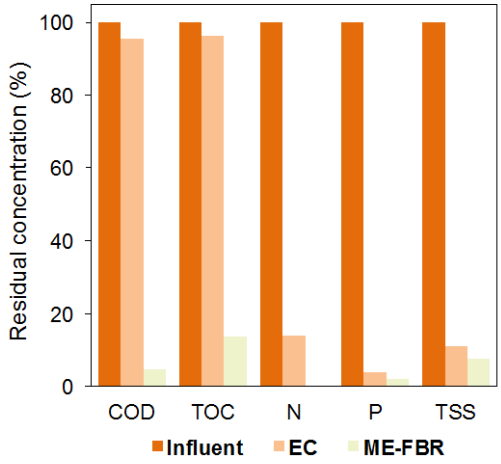

Figure 4-8: Residual concentration (as a percentage) of COD, TOC, N, P and TSS in the wastewater after the different treatments.

Electrical current was harvested just after inoculation indicating that the electroactive bacteria were already adapted to degrade the organic matter transferring the resulting electrons to the fluidized anode (Figure 4-9). This result confirmed us that EC pretreatment did not inhibit the bioelectrochemical degradation of the brewery wastewater. The current harvested reached a stationary value from the 4th day of operation of 214 A m^{-3}-bed or 25 A m$^{-3}_{wastewater}$. Regarding the cathode potential, it remained between -0.95 to -1.05 V during the experimental period. In the absence of oxygen inside the ME-FBR, at those potentials water is likely to be

reduced to form hydrogen at this electrode. The average coulombic efficiency was of 17 %, calculated over the total COD removed in the ME-FBR. The relative low coulombic efficiency in the ME-FBR may be due to several factors. Firstly, not all the electrons stored in the organic matter are available for the electroactive microorganisms, i.e. the acidogens can perform beta-oxidative reactions of long chain fatty acids, which have not been previously reported as suitable electron donors for electrogens. Secondly, electroactive bacteria compete with acetogens and methanogens for the electrons at the very last steps of the organic matter oxidation. Future research focused on studying the effects of varying the anode potential, bed expansion, the HTR or the pH could optimize the performance of the ME-FBR in terms of COD removal and current harvesting.

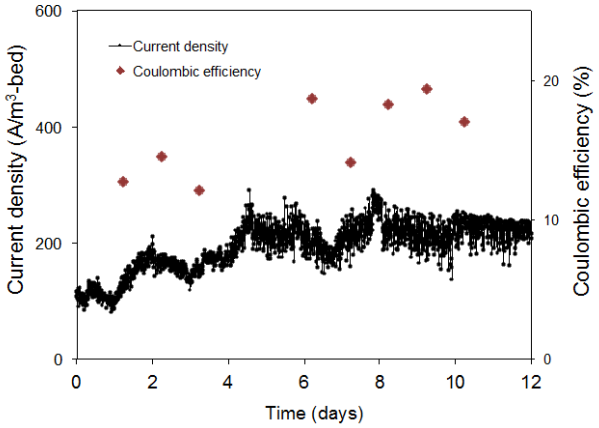

Figure 4-9: Current density production and coulombic efficiency during the operation at continuous mode of the ME-FBR.

4.11 Conclusions

By using electrocoagulation treatment before feeding the wastewater in the ME-FBR we successfully generated an effluent free of most of the insoluble matter and some refractory compounds that are hardly degraded by anaerobic microorganisms including electroactive bacteria. In addition, microorganisms present in the original primary brewery wastewater were probably removed by EC, a desired performance for the electroactive microbiome selection in the following bioreactor. This work demonstrates that merging EC with a microbial electrochemical system like ME-FBR results in an effective strategy for treating brewery wastewater. In our proposal, removal of nutrients and insoluble matter is isolated from the soluble

organic matter biodegradation stage. The economical and the environmental sustainability of the process rely on the use of the by-products generated at each stage and the minimization of the energy demand of the process. Interestingly, the energy requirements for the EC reactor have been reported to be easily powered by a renewable energy (Valero *et al.*, 2011). Regarding the by-products generated, there is a chance to recover the hydrogen generated at the cathodes of the electrochemical processes and the sludge produced at the EC, which is rich in nutrients and organic matter and could be recycled as fertilizer. Moreover, compacted aluminum waste produced in beverage industry could be used for sacrificial anodes in EC units.

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General discussion, conclusions and future work

CHAPTER 5

General discussion, conclusions and future work

The main objective of this thesis was to evaluate the potential of merging microbial electrochemical technologies (METs) with conventional reactor designs to treat wastewaters for simplifying engineering problems encountered in scaling up microbial electrochemical systems. The work presented in this thesis supports the idea that these new scenarios might present a potential alternative to classical wastewater treatment technologies and to current bioelectrochemical designs. A general discussion is presented in the framework of a question-answer session, followed by a brief section of final conclusions, recommendations and future work.

5.1 General Discussion And Conclusions

⇒ *Can a fluidized electrode act as terminal electron acceptor for electroactive bacteria?*

The biocatalysis on METs has been classically located at the electrode-biofilm interface when direct electron transfer occurs. The kinetics of the reaction can be enhanced by increasing the area of the electrodes, the activity of the microorganisms within the biofilm or by optimizing the extracellular electron transfer (EET) rate from bacteria to electrodes. Fluidized electrodes are conductive beds made of electrically conductive particles in motion that represent two relevant advantages over flat and/or static electrodes: high mass transfer-rates and large electrode surface area per unit volume. Because of these two benefits, we have transferred the concept of fluidization to the anodes of METs. The fundamental studies with *G. sulfurreducens*, acetate and a fluidized bed as an anode (Chapter 2) provided insights into the capacity of this species for utilizing a polarized electrode in motion. Firstly, we observed that a fluidized anode was a much more efficient electron discharching element for *Geobacter* cells than other suitable soluble electron acceptors. This indicated an effective bacteria-electrically conducting particle interaction and a rapid electron transfer from outermost cytochromes to the fluidized anode. In addition to utilizing a fluidized and polarized anode as final electron acceptor, *Geobacter* could efficiently couple this process of current generation with acetate oxidation, while achieving coulombic efficiencies up to 91%. When we worked with a mixed culture and a real brewery wastewater as substrate (Chapter 3), we also showed that the organic matter could be oxidized with a fluidized anode serving as final electron

acceptor. This indicates that the capacity for interacting with fluidized anodes is widespread among electroactive microbial communities.

⇒ *What is the mechanism of the electrode-bacteria interaction between Geobacter and a fluidized anode? Is this interaction viable over time?*

The use of an electrically conductive bed of particles made of a non-porous and a smooth material and with a hydrophobic nature, acting as fluidized anode, promoted the electron exchange with *G. sulfurreducens* under a planktonic state. This interaction could support growth in the same fashion as natural insoluble electron acceptors (*eg.* Fe-oxides). The nature of this interaction was based on a decoupled process involving: acetate oxidation (catabolism) and temporal electron storage in the cytochromes network followed by electron transfer to the fluidized anode (respiration). We observed that *c*-type cytochromes were involved in the process of electron storage and discharge to the fluidized anode. The cells grown in the ME-FBR showed a superior electron storage capacity in the absence of an electron acceptor in the medium than the one reported for electroactive biofilms (18-fold) (Schrott *et al.* 2011). This process supported enough inner membrane electron transfer/proton pumping for *Geobacter* to satisfy its maintenance energy requirements. The reason for this enhancement of electron storage capacity per area of electrode could be due to an increase in the number of electroactive cells, to an enhancement of the electron storage capacity per cell (increase of the total iron-binding sites on the heme network) or to both. Nevertheless, since electron storage capacity is measured with the corresponding discharging reaction, it can also be assumed that a fluidized anode polarized to 0.4 V is able to withdraw electrons stored in the cytochromes network of cells more effectively than flat and static electrodes. The phenomenon of discontinuously wiring planktonic cells in motion with a final electron acceptor suspended in the medium might allow one to develop a phenotype with a high abundance of cytochromes able to store electrons. Actually, this phenotype displayed an Fe-oxide reducing capacity 10-fold more rapid than that achieved with cells previously grown with a soluble electron acceptor such as fumarate. This is consistent with the fact that the expression of many extracytoplasmic *c* -type cytochrome genes is increased during growth with insoluble iron or electrodes versus growth on soluble TEA (Holmes *et al.*, 2006).

The viability of the interaction bacteria-fluidized anode in the ME-FBR was demonstrated by operating this system for over 2 months under a discontinuous mode with successive medium refreshments. Although the system was not tested under continuous mode, planktonic *Geobacter* has already been cultured under

continuous feeding conditions in chemostats and performing EET (Esteve-Núñez *et al.*, 2005). Actually, this strain can adapt its growth in those systems to conditions such as substrate availability and hydraulic retention time.

⇒ *What are the implications of the novel planktonic electrode-bacteria interaction based on direct extracellular electron transfer?*

To the best of our knowledge within the METs field, we have shown for the first time that *Geobacter* is able to directly transfer electrons previously stored in the heme network to a suspended polarized electrode and that this interaction is able to support growth without the need of forming a biofilm. This suggests an alternative in the paradigm of microbial EET where electroactive bacteria had to colonize an electrode in order to directly exchange electrons. By promoting the planktonic growth of *Geobacter* with the anode, every single cell is individually wired to the electrode, and using a fluidized anode made of electrically conductive microparticles maximizes this contact. The fluidized anode is a 3D discontinuous electrode made of a bed of electrically conductive individual particles that come into frequent contact in their constant motion. This interaction bacteria-electrode was possible by providing the cells with an artificial motility that conducts them towards an electron acceptor where they can discharge the electrons from acetate oxidation.

One of the main problems of METs is related to using electroactive biofilms as catalysts since this limits the extent of the reaction and the activity of cells because of the mass transport limitations. These problems can be overcome with a mediated electron transfer, or by direct electron transfer not proceeding through biofilms, where every single cell contributes to current production. The latter situation is the one found in our ME-FBR with *Geobacter* and glassy carbon anodic-particles, where the nutrients and fuel are delivered in an optimum manner. The nature of the fluidized anode and the fluid dynamics in the ME-FBR created a scenario in which attaching to the anode surface was not a strict requirement for growth. This result could be related to the fact that *Geobacter* is typically planktonic in its natural habitat, which is the subsurface and sediments, where iron oxides are its most common electron acceptor. One of the key strategies for the survival in those environments is the so-called “iron lungs”, that permit these species to temporarily maintain active electron transfer across the inner membrane in the absence of an extracellular electron acceptor. The physiological status of planktonic *Geobacter* directly reducing the fluidized anodic particles in the ME-FBR could have more common features with the one found in the reduction of disperse iron oxide particles in the subsurface than with the one found reducing flat and static anodes. In EET in anodes proceeding

through biofilms, microbes become attached to the anode, while in Fe (III)-rich environments, the pathways are more likely the opposite. This might explain the ability of ME-FBR-grown cells to rapidly reduce iron oxide.

Our ME-FBR could provide a tool for studying the physiological status of species performing EET in motion. This might yield intriguing information on how dissimilatory metal-reducing and anode-reducing microorganisms behave under different scenarios.

⇒ *What are the advantages of using a ME-FBR over other microbial electrochemical reactor designs?*

We have seen that, whereas *Geobacter* could grow planktonically in the ME-FBR with glassy carbon particles as anode, when we worked with a mixed culture and employed anodic particles made of a porous and hydrophilic material as activated carbon, a biofilm architecture was promoted from the very beginning. Fluidized bed reactors for treating wastewaters are biofilm-based configurations with a suspended bed as an electron carrier, which has several advantages.

- A large interphase contact surface between the liquid phase and the biofilm that provides good mass and heat transport properties
- Retention of biomass in small particles acting as carriers: this allows to separate the mean residence time of the liquid and the biomass
- High biomass concentration in reactors: this allows one to treat wastewater with a high organic loading or work at high rates
- The effect of fluidization of the particles eliminates bed clogging that can occur when working with packed beds
- The possibility of constant bed exchange without stopping the process

The listed advantages of fluidized bed bioreactors make them more efficient than other configurations used in wastewater treatment technology.

Because of the latter, most of these advantages can be applied to the METs field, and used for describing the benefits of using a ME-FBR with respect other bioelectrochemical configurations. The core of the ME-FBR is the fluidized electrode. The size, morphology and dynamics of the particulate electrode convert this configuration into an attractive design for any bioelectrochemical system. First of all, a fluidized electrode is 3D-element with large superficial area with an important advantage over other 3D-electrodes like packed electrodes: the fluidization state

confers good electrode-fluid mass transport properties (reduction of the diffusion boundary layer) and avoids possible clogging, commonly found when using felts, mesh and granules of carbon.

We have seen in our ME-FBR design treating real wastewater that the biofilm thickness, *ca.* 10 µm, is lower than those achieved using flat electrodes (up to 100 µm). The shear stress among the anodic particles seems governs biofilm development on the particles regarding thickness, structure and density. Thus, this opens the possibility of tuning the electroactive biofilm thickness by varying the upflow stream in the ME-FBR. An efficient MET design involves a compromise between high anode surface area and reduced biofilm thickness in order to reduce the inactive area. It has been reported that cells in the upper part of the biofilm (beyond 30-40 µm) of flat and static electrodes are most likely inactive/not growing and do not contribute to current production (Schrott *et al.* 2014). It remains unclear if higher current production can be achieved by promoting the growth of a thicker biofilm on the particles of the ME-FBR. Probably the superior mass transport conditions in a ME-FBR may allow one to develop thicker active biofilms as compared to using flat and static electrodes.

The electrochemical properties of the reactor can be improved due to the stimulation of the ion transport from the inner layers of the biofilm to the bulk liquid, and from the bulk liquid surrounding the anode to the cathode zone. This can reduce the internal resistance of the electrochemical cell, compensating the ohmic losses due to the low conductivity that is usually present in real wastewaters. In addition, we point out that the concentration overpotential can be minimized due to the good mixing properties in the ME-FBR.

Regarding reactor design, the tubular shape of our ME-FBR avoids the existence of dead zones. The reactor design is also suitable for a continuous mode of operation, and additionally provides for a compact configuration with small area requirements. Many METs require complex designs, with expensive components such as a membrane for separating anode and cathode chambers thus complicating the possibility of up-scaling the configuration. The ME-FBR is a single chamber reactor, relatively easy to operate. Other designs are modulated systems that can enhance treatment capacity by operating several modulated units in parallel or serial. However, for treating large volumes of wastewater at full-scale, these kind of configurations may not be economically viable (Zhuang *et al.* 2012).

⇒ *Controlling the microbial growth in a ME-FBR: planktonic versus biofilm*

Bacterial adhesion is a complex process that is affected by many factors, including some characteristics of the bacteria itself, the target material surface, and the environmental factors (such as the hydrodynamics or the bacteria concentration) (Donlan, 2002). In Chapter 2 and Chapter 3 and 4 (Part II) we operated different ME-FBRs. In Chapter 2, Chapter 3 and 4 (Part II) we operated different ME-FBRs. In Chapter 2, the ME-FBR used had a fluidized bed made of glassy carbon particles, the microorganisms used was a pure culture of *Geobacter sulfurreducens* and the operation of the reactor was at batch mode. Meanwhile, in Chapter 3 and 4, we combined a fluid-like bed made of activated carbon particles as anode with a mixed culture as inoculum, operated at discontinuous mode.

Cells behave as single particles in a liquid culture, and the rate of association with the fluidized particles depends largely on the velocity characteristics of the recirculating liquid in the ME-FBR. Typically, a fluid motion in the bulk liquid favors bacteria adhesion because of the enhancement of the cell transportation to the surface by convection (Rijnaarts *et al.*, 1993). However, shear forces induced by moving beads in mixed systems can also reduce adhesion and provoke cell detachment. In order to fluidize the bed, large linear velocities of the liquid are required, and thus cells are subject to a great turbulence and mixing. The hydrodynamic conditions in all the ME-FBRs used in this work were similar; therefore, the author will not discuss the possible influence of this parameter on the growth of cells under planktonic or biofilm mode in the different ME-FBR.

Regarding the influence of the material surface of the fluidized anodic particles, the use of glassy carbon particles as fluidized anode promoted the growth of planktonic cells performing direct EET with the fluidized anode was promoted from the beginning (Chapter 2). The surface of the glassy carbon particles presented poor roughness, very low superficial imperfections and pores, what reduces the anchoring elements for bacteria. The glassy carbon is a material with a relative hydrophobicity nature and low wettability, what complicates the attachment and growth of cells. In addition, the glassy carbon particles did not show the typical surface functional groups described elsewhere as key in the bacteria-electrode interaction (Fiset and Puig, 2015). Moreover, it has been reported that hydrophobic surfaces require larger periods of time to be colonized by microbes than hydrophilic surfaces in MFCs (Santoro *et al.*, 2014). In fact, it has been observed that the nature of the electrode surface could change to a hydrophilic surface in presence of wastewater by promoting a fast biofilm formation on it. We hypothesize that *Geobacter*, under the

presence of a polarized anode with hydrophobic surface and a high turbulence in the bulk media, developed alternative strategies to respire such a polarized particles in motion. Thus, the planktonic growth of *Geobacter* cells could have been a survival strategy of this bacterial strain to maintain the metabolic activity. After 1 month of operating the ME-FBR with glassy carbon particles, we did not observe any biofilm development on their surface.

In contrast, in Chapter 3 and Chapter 4 (Part II) we used activated carbon particles as fluidized bed, a material with high porosity and with charged functional groups bounded at its surface that confers it a hydrophilic nature. We observed a rapid colonization of the particles, although a mature biofilm (*ca.* 10 µm thickness) was not formed until several months of operation at continuous mode. The cell attachment occurred firstly at the pores and cavities of the material, the regions sheltered from hydraulic shear forces and the shear stress from particle-to-particle attrition. We hypothesize that the nature of the surface of the activated carbon particles promoted the cell attachment easily, allowing the electroactive bacteria to be permanently in contact with the anode, a terminal electron acceptor as typically occurs under a fixed bed scenario. In these studies we used a mixed culture from activated sludge as inoculum, a factor that can also influence the formation of biofilms since bacterial attachment to a surface depends on the composition of the attaching population and can considerably differ from the attachment of the species in pure culture (McEldowney and Fletcher, 1987).

The hydraulic conditions in the fluidized reactors strongly influence the particles colonization. Operating at continuous mode often promotes rapid biomass attachment to carriers in order to avoid cell washout, and the hydraulic retention time can strongly influence the rate of particles colonization. This operating mode might stimulate as well the development of a biofilm on the fluidized activated carbon particles in the ME-FBR that treated brewery wastewater. In contrast, when operating the glassy carbon ME-FBR at batch or discontinuous mode, cells did not attach to the carrier and the biomass remained actively planktonic while respiring the electrode in motion.

We believe that both electroactive planktonic and biofilm-forming bacteria performing EET can coexist in a ME-FBR, and they might exhibit a distinct phenotype with respect to gene transcription and growth rate. Although in METs the catalysis is typically located at the biofilm developed on the electrode (usually static), planktonic growth may be promoted by creating different specific scenarios based on the nature of the electrode surface.

⇒ *Does a ME-FBR represent a suitable technology for treating industrial wastewaters? What are the advantages of using a ME-FBR over conventional wastewater treatment technologies? Can it compete with anaerobic digesters?*

In Chapter 3 we studied the performance of a ME-FBR and of a classical anaerobic fluidized bed reactor with non-electrically conducting particles (biolite) on the treatment of a real brewery wastewater. We showed that the bioelectrochemical system could efficiently remove most of the organic matter from the brewery wastewater (> 75 %), operating at different organic loading rates (OLR). Maximum current densities reached values of 210 A m^{-3} bed, which was estimated to be 0.1 A m^{-2} (this calculation assumes spherical particles). This value is low as was expected for the treatment of a real wastewater as compared to the current densities that were obtained with acetate-fed systems. The coulombic efficiencies values ranged from 10 to 55 %, decreasing gradually as the organic loading rate was enhanced. A chemical oxygen demand (COD) removal of 88 % was achieved treating a brewery effluent at al OLR of 1.74 kg COD m^{-3} reactor d^{-1}. In fact, the ME-FBR was in general able to operate at removal rates greater than 1.5 kg COD $m^{-3}_{reactor}$ d^{-1}, outperforming other proposed METs for treating this kind of wastewater (Dong *et al.*, 2015; Wang *et al.*, 2008; Zhuang *et al.*, 2012a). However, we note that this rate is still low if we compare it to operating conditions of high-rate anaerobic digesters (AD). The digester, being a direct competitor of METs in wastewater treatment, can treat from 5 to 25 kg COD $m^{-3}_{reactor}$ d^{-1} (Pham *et al.*, 2006).

Increasing the OLR of the ME-FBR provoked a drop of the coulombic efficiency, favoring the development of methanogenesis over the electrogenic pathway. Thus, it seems that if this reactor were able to treat wastewater at similar bioconversion rates as that of AD, the fraction of organic matter degraded through current generation would be practically insignificant. This represents a real drawback for this technology and therefore improving the coulombic efficiencies of the process is critical for this technology to be a real alternative to AD. However, instead of being a substitute, the ME-FBR might be considered as a complementary technology. The microbial electrochemical treatment maintains several advantages over AD, such as the possibility of directly collecting electricity, the capability of these systems for treating low concentration substrates, and the capacity of operating at a wider range of temperatures. Therefore, the ME-FBR could, for instance, treat the effluent of an anaerobic digester, achieving high coulombic efficiencies and recovering electricity in the form hydrogen.

In our study in Chapter 3, the MET configuration clearly outperformed the biolite-M-FBR (anaerobic digester) for all of the OLRs tested. The biolite-M-FBR COD removals were below 64% and decreased as the ORL was enhanced. The reasons for this marked difference in the performance of both reactors seemed to be related to a faster and higher growth and colonization of electroactive bacteria in the conductive and polarized particles as compared to the methanogenic community attached to the biolite particles. The higher quantity of biomass on the bed of the ME-FBR led to a faster degradation of organic matter in this reactor and a higher VFAs consumption. The enrichment of the polarized activated carbon particles with *Geobacter* species (*ca.* 20%), the model bacteria in microbial electrochemistry, together with the current generation in the ME-FBR, suggested that the fluidized bed was acting as an electron acceptor for a fraction of the microbial community. We hypothesize that this role of the bed could be responsible for the faster and higher colonization of the activated carbon as compared to biolite particles. The electrical conducting nature of the carrier in the ME-FBR could also have stimulated syntrophic relations between different microbial communities through direct interspecies electron transfer. It is known that *Geobacter* and methanogenic species can exchange electrons (Rotaru *et al.*, 2014). The mere presence of the conductive bed in the ME-FBR could have enhanced methane production by promoting biological electrical connections between microbial species, feeding electrons to methanogens for the reduction of carbon dioxide to methane.

It remains unclear whether the biolite-M-FBR needed a higher start-up period than the ME-FBR and, after this period of time, similar organic removal rates could have been achieved. Our results suggest that by using a fluidized anode one can accelerate the start-up phase of fluidized bed reactors, which commonly require several months to obtain a mature biofilm. In this sense, the ME-FBR could compete with the anaerobic digester in areas other than in the rate of substrate conversion such as providing operating advantages as process stability or reduced start-up periods. Further studies in this area of investigation are likely to provide valuable information that may open new niche applications of the ME-FBR.

Another aspect to be considered is the energy and/or sub-products recovery of the treatment process. The current density produced in the ME-FBR can be recovered as H_2 formed at the cathode with the electrons coming from the organic matter oxidation on the fluidized anode. We estimated a value of *ca.* 0.2 $L_{H_2}\ L^{-1}_{reactor}\ d^{-1}$ (assuming a 80% efficiency, 1 atm, 25ºC), which is comparable to the output values reported in previous studies with real wastewater (Cusick *et al.*, 2011; Verea *et al.*, 2014).

The ME-FBR showed to be an effective technology for removing most of the organic matter from a brewery wastewater. However, this system cannot completely treat this wastewater, and only the fraction of nutrients that are consumed in microbial anabolism is removed. As is the case with anaerobic digesters, it becomes necessary to complement the ME-FBR with other unit operations in which nutrients are removed. Other designs of METs could have a role in eliminating phosphorous or nitrogen as was described in the introductory section of this thesis.

⇒ *Can a MET with an activated-sludge-like configuration be used as a post-treatment system for removing nitrogen? What are the parameters that affect the system's performance?*

METs have been shown to stimulate the organic matter degradation by supplying electroactive microorganisms to the anode as a final electron acceptor. While the degradation of organic matter in brewery effluents in bioelectrochemical systems has been extensively studied, the removal of the nutrients has not yet been addressed. One of the systems that we explored in this thesis for removing nutrients consisted of a hybrid-MET that simulated a 2-chamber activated-sludge system. In Chapter 4 of this thesis we showed that it is possible to reuse or convert a classical activated sludge reactor into a membrane-free MET reactor for removing nitrogen and carbon from low organic matter wastewater, and with no external aeration. This kind of reactor could be employed as a post-treatment of effluents coming from a previous organic carbon removal step, which is unable to remove nutrients, like a ME-FBR (effluent from 50 to 300 mg COD L^{-1}) or an anaerobic digester.

The nitrogen removal was stimulated due to the presence of a polarized biocathode, which acted as electron source for denitrifying microorganisms. The ammonium was oxidized by aerobic microorganisms at low DO concentrations (down to 0.3-0.8 mg O_2 L^{-1}), which were mainly enriched in the second chamber of the system and attached to the counter electrode (anode). We did not detect any significant influence of the anode on that reaction. The influence of the COD/N (mass) ratio of the influent on the system performance was studied. At the highest ratio tested (4), the nitrification step slightly limited the system's capacity to remove nitrogen (87 % of nitrification efficiency). Nevertheless, the highest nitrogen removal in our reactor, 81 % and 19 g NO_3^--N m^{-3}-TCC day^{-1}, was achieved at this ratio. Low concentrations of organic matter in the influent (ratio of COD/N=2) did not favor the bioelectrochemical reduction of nitrate at the cathode because the levels of dissolved oxygen (DO) in the media inhibited this process. Since current density consumption and the total N removal tended to increase at higher COD/N ratios, the utilization of

the cathode as an electron donor by denitrifying microorganisms was favored. The assays at OCP and in the absence of electrodes showed that the presence of the electrodes and their polarization were necessary for achieving high nitrogen removal. The analysis of the effect of cathode potential on the nitrogen removal showed that denitrification was favored at low working electrode (WE) potentials. In contrast, the nitrification step limited the system's capacity to remove nitrogen when the system was operated at the lowest WE potentials tested. The reason of this seemed to be the fast electrochemical reduction of the dissolved oxygen in the medium, depriving the ammonium-oxidizing bacteria of sufficient levels of electron acceptor. We could characterize further our hybrid-MET by using additional techniques to qualitatively study the microbial community enriched in our electrodes. From thermogravimetric analysis, we observed that a thick biofilm was colonizing the cathode, and from the SEM imaging showed that this film was stratified. Bacillus shaped microorganism were found in intimate contact with the carbon fibbers of the WE. The microbial community analysis gave us more information about the metabolic activities being performed in each chamber. Denitrifying genera were enriched in the WE, whereas ammonium oxidizing bacteria and nitrite oxidizing bacteria were more abundant at the auxiliar electrode (anode). Deltaproteobacteria, a class that has been typically detected in microbial electrochemical systems (Hochstrat *et al.*, 2015), were enriched in the WE of our system.

One the main benefits of utilizing METs for removing nitrogen is the low sludge produced in the denitrification step as compared to heterotrophic denitrification. Our system actually generated a 3-fold less of a sludge volume as produced in a similar configuration using an activated sludge treatment of a settled wastewater mixed with synthetic wastewater.

In Chapter 4 we operated this air-free hybrid configuration as a proof of concept of an alternative method to remove nutrients from effluents having low levels of organic matter. For achieving an economic viable operation of this system, it is necessary to minimize the internal resistance and the electrode over-potential (optimization of its area and material) so that the energy demanded by the potentiostat or power source can be competitive with the energy required by aerated systems. In contrast, the extra costs associated with the management of the sludge produced are highly reduced with our proposed system. We believe that it can be advantageous to adapt METs to already built reactors and integrate these systems into urban wastewater treatment plants for gaining experience at a large-scale with METs, valuable for developing optimized designs of these systems.

⇒ *Is electrocoagulation a suitable pre-treatment for removing the suspended solids and nutrients from a brewery effluent?*

EC is an efficient process for removing TSS and nutrients from food industry wastewaters (Meas *et al.*, 2010; Valero *et al.*, 2011). Integrating EC as a pretreatment for the wastewater fed to a ME-FBR provides the opportunity of eliminating most of the nutrients and solids before they are hydrolyzed and solubilized in the bulk liquid. We showed in Chapter 5 that by using EC, nutrients and suspended solids could efficiently be removed from a brewery wastewater directly collected from the homogenization tank (up to 96 % of the total suspended solids (TSS), 20 % of COD and 98 % of the nutrients (N and P) were removed).

We analyzed the effect of reaction time (RT) and applied current density (*j*) on the removal of the TSS, total nitrogen (TN), total phosphorous (TP) and COD. Under the conditions tested in our study, the RT highly affected the performance of the reactor, especially the removal of TSS and nutrients. We hypothesize that working at higher RTs allowed the electro-generation of a larger amount of coagulant and favored the formation of flocs. In contrast, varying *j* did not significantly affect the removal of suspended matter. Enhancing the RT had a minor impact on the power consumption of the reactor, whereas increasing *j* triggered it.

One the advantage of EC technology is that the generated effluent is free of most of the insoluble matter and some refractory compounds that are hardly degraded by anaerobic microorganisms including electroactive bacteria. This avoids the need of a post-treatment to remove these kinds of compounds.

⇒ *Can EC complement a ME-FBR for achieving a complete wastewater treatment?*

The limitations of METs for completely treating wastewaters, as was seen in Chapter 3 with the ME-FBR, suggest the need for supporting these systems with complementary technologies for post-treatment (Chapter 4, Part I) or pre-treatment (Chapter 4, Part II). In both studies, the removal of nutrients is isolated from the soluble organic matter biodegradation stage. This allows one to work under optimum conditions for each stage.

We showed that the effluent from the EC step could successfully be further treated in a ME-FBR for removing the soluble organic matter (*ca.* 87 %), coupling oxidation (17 %) to current generation (214 A m^{-3}-bed). This demonstrated the compatibility of these two technologies based on electrode-mediated reactions. In addition, the ME-FBR also removed more than 50 % of the TSS that remained in the

wastewater after the EC process. Overall, we can conclude that by merging EC technology with a microbial electrochemical system like ME-FBR, one results in an effective strategy for completely treating brewery wastewater. This tandem of electrochemical technologies can be a flexible and versatile platform for treating wastewaters. The reaction time and the current density circulating through the EC reactor could be fine-tuned to obtain different qualities of the effluent (TSS, TN and TP). Varying the operating parameters such as HRT, anode potential or bed expansion could provide as well a tool for controlling the effluent quality of the ME-FBR treatment.

The full process (EC+ ME-FBR) is a perfect example of a strategy in context of the water-energy nexus. While treating wastewater, there is a chance to recover energy in the form of H_2 on the two stages (generated at the cathodes from electrolytic dissociation of water). The theoretical estimated values of H_2 collection that can be taken as maximum benchmark are of *ca.* 0.2 L $_{H_2}$ $L^{-1}_{reactor}$ d^{-1} for the ME-FBR, and *ca.* 0.11 L $_{H_2}$ $L^{-1}_{reactor}$ (4.2 L $_{H_2}$ h^{-1}) for each batch of 15 min in the EC (1.5 L of wastewater) at j=2.6 mA cm^{-1}. In addition to the possibility of producing energy as revenue to compensate the operational cost, the nutrients and solids of the sludge produced at the EC can be recovered and reused as fertilizer. Our proposal of merging EC with ME-FBRs provides a new approach for brewery wastewater treatment while offering a valuable alternative to energy generation and sub-product recovery.

5.2 Final Conclusions

The general conclusions that can be withdrawn from this thesis are the following:

- Results demonstrate that a fluidized anode is a suitable final electron acceptor for electroactive bacteria able to support growth.
- We described a new kind of DEET based on the planktonic interaction of *Geobacter* cells with a fluidized anode in a ME-FBR that demonstrates that forming a biofilm is not a strict requirement for bacteria to grow performing DEET.
- A ME-FBR is a suitable technology for effectively degrading the organic matter of brewery wastewater and that outperforms a conventional M-FBR at least during the start-up period. However, the parameters of this

configuration need to be optimized to allow this technology to be competitive with respect to anaerobic digesters.

- A membrane free hybrid-MET based on an activated sludge configuration is an effective configuration for removing nitrogen and organic matter from low COD effluents.
- Our results demonstrate that electrocoagulation is an effective method for removing the TSS, TN and TP of a brewery effluent that allows one to easily tune these concentrations in the final effluent.
- Integrating EC as pre-treatment of ME-FBRs results in an effective strategy for completely treating a brewery wastewater. The process needs to be further validated and developed for improving the treatment capacity and the efficiency of the system.

5.3 Recommendations And Future Work

Recommendations for further research are made based on the results exposed in this thesis. Some limitations should be overcome and optimization of an operational configuration should be addressed prior to using METs for real world applications.

a) In order to obtain a better understanding of the electron transfer process occurring between fluidized anodes and Geobacter a number of additional assays should be performed:

A deeper analysis concerning the changes in *Geobacter* physiology while performing continuous charging-discharging processes in the ME-FBR could provide further information. Questions such as how *Geobacter* is able to store electrons for energy maintenance during relatively large periods of time, or what are the common mechanisms for EET to fluidized anodes and iron oxides and under which conditions are those pathways expressed, should be addressed. These questions are critical for understanding the biological mechanisms behind the bacteria-fluidized electrode interaction.

The assays should be conducted towards elucidating the key proteins involved in the electron transfer from the planktonic cells to the fluidized anode. The analysis of the parameters that affect and promote the planktonic cell growth, and the biofilm development in the ME-FBR could provide insights into the mechanisms of EET in the microbial electrochemistry and in geochemistry field.

b) In order to scale-up ME-FBRs technology with a complementary system as a real wastewater treatment tool, the main bottlenecks that need to be overcome are:

The methanogenic pathway in the ME-FBR should be avoided or minimized. For this purpose, the following strategies should be evaluated:

- Researching focused on the study of new microorganism that could better adapt to respiring a fluidized anode in ME-FBR systems. So far, *Geobacter* was used as model microorganism in biofilm-based systems. However, the search for new bacteria that could interact more efficiently with fluidized electrodes, either directly or with mediators, could help on optimizing the rate of the EET.
- Screening key parameters that stimulate the methanogenic pathway over the electrogenic ones. The effect on both the treatment efficiency and current production of parameters such as fluidized anode potential, bed expansion, pH or recirculating flux should be studied in depth in the ME-FBR to maximize bioelectrochemical organic matter removal. The use of methanogenesis inhibitors might aid in this regard.
- A study should be performed focused on developing the biofilm on the fluidized activated carbon particles. The strength of the shearing forces governs the quantity and quality of anode colonization. The thickness of the electroactive and non-electroactive biofilm is a key parameter affecting the efficiency of the treatment. An optimum biofilm thickness should be a compromised between quantity of biomass and its metabolic state. A possible correlation amongst the thickness of the biofilm, coulombic efficiency and the enriched microbial community would bring useful information for promoting the electrogenic pathway over the methanogenic one.

The energy consumption of the ME-FBR, of the 2-chamber hybrid-MET for removing nitrogen and of the EC treatment should be minimized. Most of this energy is that required by the potentiostat or power source needed for operating the technologies proposed in this thesis. In this fashion:

- A study focused on identifying the main terms contributing to the internal resistance of the reactor should be performed. By utilizing more electrically conductive materials as electrodes, one could help to reduce the electrode over-potential caused by mass and charge transfer limitations. Reducing the

distance between the electrodes in the 3 configurations used in this thesis might aid in reducing the electrolyte resistance. Furthermore, by testing different reactor architectures it might be possible to increase ion transport and therefore reactor efficiency. However, little can be done with respect to the low conductivity of wastewaters. Adding external sources of ions does not seem adequate since it supposes a secondary pollution of the wastewater.

The post-treatment and pre-treatments systems proposed in this thesis need to be further validated and developed as ME-FBR-compatible and efficient technologies for treating real wastewater. In this regard:

- Real wastewater, as was demonstrated in the ME-FBR, should be tested as substrate in the 2-chamber membrane free hybrid-MET. The study presented in Chapter 4, Part I, is a proof-of concept analysis performed with acetate as substrate and ammonium as the nitrogen form. More realistic information would be obtained using a more complex source of organic matter. In this situation, a new study elucidating the nature of the enriched microbial communities on the electrodes should be performed since probably a wider diversity of microorganisms may appear. FISH analysis could help to characterize the stratification of the biofilm developed on the working electrode of this system. An interesting study would be to make a carbon balance in the system so as to determine the extent of autotrophic nitrogen removal and that of heterotrophic removal.
- The stability and robustness of both the EC and the 2-chamber hybrid-MET under different perturbations, like nutrients and organic matter load peaks, or an influent dilution, should be tested.

References

References

Aguirre-Sierra A., Reija A., Berná A., Salas J.J., and Esteve-Nuñez A. (2014). Microbial Electrochemical Constructed Wetlands (METlands): design and operation conditions for enhancing the removal of pollutants in real urban wastewater.

Allen, J.W.A., Sawyer, E.B., Ginger, M.L., Barker, P.D., and Ferguson, S.J. (2009). Variant c-type cytochromes as probes of the substrate specificity of the E. coli cytochrome c maturation (Ccm) apparatus. Biochem J *419*, 177–184.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. *56*, 1919–1925.

Angosto, J.M., Fernández-López, J.A., and Godínez, C. (2015). Brewery and liquid manure wastewaters as potential feedstocks for microbial fuel cells: a performance study. Environ. Technol. *36*, 68–78.

Annachhatre, A.P. (1996). Anaerobic treatment of industrial wastewaters. Resour. Conserv. Recycl. *16*, 161–166.

Aronesty, E. (2011). ea-utils : "Command-line tools for processing biological sequencing data."

Arredondo, M.R., Kuntke, P., Jeremiasse, A.W., Sleutels, T.H.J.A., Buisman, C.J.N., and Heijne, A. ter (2015). Bioelectrochemical systems for nitrogen removal and recovery from wastewater. Environ. Sci. Water Res. Technol. *1*, 22–33.

Badalamenti, J.P., Krajmalnik-Brown, R., and Torres, C.I. (2013). Generation of High Current Densities by Pure Cultures of Anode-Respiring Geoalkalibacter spp. under Alkaline and Saline Conditions in Microbial Electrochemical Cells. mBio *4*.

Balaguer, M.D., Vicent, M.T., and Parfs, J.M. (1997). A Comparison of Different Support Materials in Anaerobic Fluidized Bed Reactors for the Treatment of Vinasse. Environ. Technol. *18*, 539–544.

Baranitharan, E., Khan, M.R., Yousuf, A., Teo, W.F.A., Tan, G.Y.A., and Cheng, C.K. (2015). Enhanced power generation using controlled inoculum from palm oil mill effluent fed microbial fuel cell. Fuel *143*, 72–79.

Baytshtok, V., Lu, H., Park, H., Kim, S., Yu, R., and Chandran, K. (2009). Impact of varying electron donors on the molecular microbial ecology and biokinetics of methylotrophic denitrifying bacteria. Biotechnol. Bioeng. *102*, 1527–1536.

Behera, M., Jana, P.S., More, T.T., and Ghangrekar, M.M. (2010). Rice mill wastewater treatment in microbial fuel cells fabricated using proton exchange membrane and earthen pot at different pH. Bioelectrochemistry *79*, 228–233.

Bereza-Malcolm, L.T., Mann, G., and Franks, A.E. (2015). Environmental Sensing of Heavy Metals Through Whole Cell Microbial Biosensors: A Synthetic Biology Approach. ACS Synth.

Biol. *4*, 535–546.

Berk, R.S., and Canfield, J.H. (1964). Bioelectrochemical Energy Conversion. Appl. Microbiol. *12*, 10–12.

Bigalke, J., and Grabner, E.W. (1997). The Geobattery model: a contribution to large scale electrochemistry. Electrochimica Acta *42*, 3443–3452.

Blanchet, E., Desmond, E., Erable, B., Bridier, A., Bouchez, T., and Bergel, A. (2015). Comparison of synthetic medium and wastewater used as dilution medium to design scalable microbial anodes: Application to food waste treatment. Bioresour. Technol. *185*, 106–115.

Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., and Caporaso, J.G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods *10*, 57–59.

Bond, D.R., and Lovley, D.R. (2003). Electricity production by Geobacter sulfurreducens attached to electrodes. Appl. Environ. Microbiol. *69*, 1548–1555.

Bond, D.R., Holmes, D.E., Tender, L.M., and Lovley, D.R. (2002). Electrode-reducing microorganisms that harvest energy from marine sediments. Science *295*, 483–485.

Bond, D.R., Strycharz-Glaven, S.M., Tender, L.M., and Torres, C.I. (2012). On Electron Transport through Geobacter Biofilms. ChemSusChem *5*, 1099–1105.

Borjas, Z., Ortiz, J.M., Aldaz, A., Feliu, J., and Esteve-Núñez, A. (2015). Strategies for Reducing the Start-up Operation of Microbial Electrochemical Treatments of Urban Wastewater. Energies *8*, 14064–14077.

Borjas Z. (2016). Physiological and Operation Strategies for Optimizing Geobacter–based Electrochemical Systems. Alcalá de Henares.

Bretschger, O., Obraztsova, A., Sturm, C.A., Chang, I.S., Gorby, Y.A., Reed, S.B., Culley, D.E., Reardon, C.L., Barua, S., Romine, M.F., *et al.* (2007). Current production and metal oxide reduction by Shewanella oneidensis MR-1 wild type and mutants. Appl Env. Microbiol *73*, 7003–7012.

Brown, R.K., Harnisch, F., Dockhorn, T., and Schröder, U. (2015). Examining sludge production in bioelectrochemical systems treating domestic wastewater. Bioresour. Technol. *198*, 913–917.

Busalmen, J.P., Esteve-Nunez, A., Berna, A., and Feliu, J.M. (2008). C-type cytochromes wire electricity-producing bacteria to electrodes. Angew Chem Int Ed Engl *47*, 4874–4877.

Busalmen, J.P., Esteve-Nunez, A., Berna, A., and Feliu, J.M. (2010). ATR-SEIRAs characterization of surface redox processes in G. sulfurreducens. Bioelectrochemistry *78*, 25–29.

Butler, J.E., Glaven, R.H., Esteve-Nunez, A., Nunez, C., Shelobolina, E.S., Bond, D.R., and Lovley, D.R. (2006). Genetic characterization of a single bifunctional enzyme for fumarate

reduction and succinate oxidation in Geobacter sulfurreducens and engineering of fumarate reduction in Geobacter metallireducens. J Bacteriol *188*, 450–455.

Caccavo, F., Jr., Lonergan, D.J., Lovley, D.R., Davis, M., Stolz, J.F., and McInerney, M.J. (1994). Geobacter sulfurreducens sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl Env. Microbiol *60*, 3752–3759.

Call, D., and Logan, B.E. (2008). Hydrogen production in a single chamber microbial electrolysis cell lacking a membrane. Env. Sci Technol *42*, 3401–3406.

Cañizares, P., Carmona, M., Lobato, J., Martínez, F., and Rodrigo, M.A. (2005). Electrodissolution of Aluminum Electrodes in Electrocoagulation Processes. Ind. Eng. Chem. Res. *44*, 4178–4185.

Cao, X., Huang, X., Liang, P., Xiao, K., Zhou, Y., Zhang, X., and Logan, B.E. (2009). A new method for water desalination using microbial desalination cells. Environ. Sci. Technol. *43*, 7148–7152.

Cao, X., Song, H., Yu, C., and Li, X. (2015). Simultaneous degradation of toxic refractory organic pesticide and bioelectricity generation using a soil microbial fuel cell. Bioresour. Technol. *189*, 87–93.

Capodaglio, A.G., Molognoni, D., Dallago, E., Liberale, A., Cella, R., Longoni, P., Pantaleoni, L., Capodaglio, A.G., Molognoni, D., Dallago, E., *et al.* (2013). Microbial Fuel Cells for Direct Electrical Energy Recovery from Urban Wastewaters, Microbial Fuel Cells for Direct Electrical Energy Recovery from Urban Wastewaters. Sci. World J. Sci. World J. *2013, 2013*, e634738.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods *7*, 335–336.

Carbajosa, S., Malki, M., Caillard, R., Lopez, M.F., Palomares, F.J., Martín-Gago, J.A., Rodríguez, N., Amils, R., Fernández, V.M., and De Lacey, A.L. (2010). Electrochemical growth of Acidithiobacillus ferrooxidans on a graphite electrode for obtaining a biocathode for direct electrocatalytic reduction of oxygen. Biosens. Bioelectron. *26*, 877–880.

Carmona-Martínez, A.A., Pierra, M., Trably, E., and Bernet, N. (2013a). High current density via direct electron transfer by the halophilic anode respiring bacterium Geoalkalibacter subterraneus. Phys. Chem. Chem. Phys. PCCP *15*, 19699–19707.

Carmona-Martínez, A.A., Harnisch, F., Kuhlicke, U., Neu, T.R., and Schröder, U. (2013b). Electron transfer and biofilm formation of Shewanella putrefaciens as function of anode potential. Bioelectrochemistry Amst. Neth. *93*, 23–29.

Cercado-Quezada, B., Delia, M.-L., and Bergel, A. (2010). Testing various food-industry wastes for electricity production in microbial fuel cell. Bioresour. Technol. *101*, 2748–2754.

Çetinkaya, A.Y., Köroğlu, E.O., Demir, N.M., Baysoy, D.Y., Özkaya, B., and Çakmakçı, M. (2015). Electricity production by a microbial fuel cell fueled by brewery wastewater and the

factors in its membrane deterioration. Chin. J. Catal. *36*, 1068–1076.

Chandrasekhar, K., and Venkata Mohan, S. (2012). Bio-electrochemical remediation of real field petroleum sludge as an electron donor with simultaneous power generation facilitates biotransformation of PAH: effect of substrate concentration. Bioresour. Technol. *110*, 517–525.

Chang, H.T., Rittmann, B.E., Amar, D., Heim, R., Ehlinger, O., and Lesty, Y. (1991). Biofilm detachment mechanisms in a liquid-fluidized bed. Biotechnol. Bioeng. *38*, 499–506.

Chen, Y., Cheng, J.J., and Creamer, K.S. (2008). Inhibition of anaerobic digestion process: A review. Bioresour. Technol. *99*, 4044–4064.

Chernicharo, C. a. L. (2006). Post-Treatment Options for the Anaerobic Treatment of Domestic Wastewater. Rev. Environ. Sci. Biotechnol. *5*, 73–92.

Childers, S.E., Ciufo, S., and Lovley, D.R. (2002). Geobacter metallireducens accesses insoluble Fe(iii) oxide by chemotaxis. Nature *416*, 767–769.

Choi, C., and Hu, N. (2013). The modeling of gold recovery from tetrachloroaurate wastewater using a microbial fuel cell. Bioresour. Technol. *133*, 589–598.

Chun, C.L., Payne, R.B., Sowers, K.R., and May, H.D. (2013). Electrical stimulation of microbial PCB degradation in sediment. Water Res. *47*, 141–152.

Ciudad, G., Werner, A., Bornhardt, C., Muñoz, C., and Antileo, C. (2006). Differential kinetics of ammonia- and nitrite-oxidizing bacteria: A simple kinetic study based on oxygen affinity and proton release during nitrification. Process Biochem. *41*, 1764–1772.

Clauwaert, P., Rabaey, K., Aelterman, P., de Schamphelaire, L., Pham, T.H., Boeckx, P., Boon, N., and Verstraete, W. (2007). Biological denitrification in microbial fuel cells. Env. Sci Technol *41*, 3354–3360.

Clauwaert, P., Tolêdo, R., van der Ha, D., Crab, R., Verstraete, W., Hu, H., Udert, K.M., and Rabaey, K. (2008). Combining biocatalyzed electrolysis with anaerobic digestion. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res. *57*, 575–579.

Coates, J.D., Ellis, D.J., Blunt-Harris, E.L., Gaw, C.V., Roden, E.E., and Lovley, D.R. (1998). Recovery of Humic-Reducing Bacteria from a Diversity of Environments. Appl. Environ. Microbiol. *64*, 1504–1509.

Coma, M., Puig, S., Pous, N., Balaguer, M.D., and Colprim, J. (2013). Biocatalysed sulphate removal in a BES cathode. Bioresour. Technol. *130*, 218–223.

Cruz Viggi, C., Rossetti, S., Fazi, S., Paiano, P., Majone, M., and Aulenta, F. (2014). Magnetite particles triggering a faster and more robust syntrophic pathway of methanogenic propionate degradation. Environ. Sci. Technol. *48*, 7536–7543.

Cruz Viggi, C., Presta, E., Bellagamba, M., Kaciulis, S., Balijepalli, S.K., Zanaroli, G., Petrangeli Papini, M., Rossetti, S., and Aulenta, F. (2015). The “Oil-Spill Snorkel”: an innovative bioelectrochemical approach to accelerate hydrocarbons biodegradation in marine

sediments. Microbiotechnology Ecotoxicol. Bioremediation 881.

Cusick, R.D., and Logan, B.E. (2012). Phosphate recovery as struvite within a single chamber microbial electrolysis cell. Bioresour. Technol. *107*, 110–115.

Cusick, R.D., Bryan, B., Parker, D.S., Merrill, M.D., Mehanna, M., Kiely, P.D., Liu, G., and Logan, B.E. (2011). Performance of a pilot-scale continuous flow microbial electrolysis cell fed winery wastewater. Appl. Microbiol. Biotechnol. *89*, 2053–2063.

Cusick, R.D., Kim, Y., and Logan, B.E. (2012). Energy Capture from Thermolytic Solutions in Microbial Reverse-Electrodialysis Cells. Science *335*, 1474–1477.

Cusick, R.D., Ullery, M.L., Dempsey, B.A., and Logan, B.E. (2014). Electrochemical struvite precipitation from digestate with a fluidized bed cathode microbial electrolysis cell. Water Res. *54*, 297–306.

Daulton, T.L., Little, B.J., Jones-Meehan, J., Blom, D.A., and Allard, L.F. (2007). Microbial reduction of chromium from the hexavalent to divalent state. Geochim. Cosmochim. Acta *71*, 556–565.

Dávila, D., Esquivel, J.P., Sabaté, N., and Mas, J. (2011). Silicon-based microfabricated microbial fuel cell toxicity sensor. Biosens. Bioelectron. *26*, 2426–2430.

Deeke, A., Sleutels, T.H.J.A., Donkers, T.F.W., Hamelers, H.V.M., Buisman, C.J.N., and Ter Heijne, A. (2015). Fluidized Capacitive Bioanode As a Novel Reactor Concept for the Microbial Fuel Cell. Environ. Sci. Technol. *49*, 1929–1935.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G.L. (2006). Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl. Environ. Microbiol. *72*, 5069–5072.

Dewan, A., Beyenal, H., and Lewandowski, Z. (2008). Scaling up microbial fuel cells. Env. Sci Technol *42*, 7643–7648.

Díaz, E. (2008). Microbial Biodegradation: Genomics and Molecular Biology (Horizon Scientific Press).

Dominguez-Garay A. (2016). Bioelectrochemically-assisted remediation: a novel strategy for cleaning-up polluted soils. Alcalá de Henares.

Dong, Y., Qu, Y., He, W., Du, Y., Liu, J., Han, X., and Feng, Y. (2015). A 90-liter stackable baffled microbial fuel cell for brewery wastewater treatment based on energy self-sufficient mode. Bioresour. Technol. *195*, 66–72.

Donlan, R.M. (2002). Biofilms: Microbial Life on Surfaces. Emerg. Infect. Dis. *8*, 881–890.

Du, Z., Li, H., and Gu, T. (2007). A state of the art review on microbial fuel cells: A promising technology for wastewater treatment and bioenergy. Biotechnol Adv *25*, 464–482.

Eaton, A.D., and Franson, M.A.H. (2005). Standard Methods for the Examination of Water & Wastewater (American Public Health Association).

Edgar, R.C. (2010). Search and clustering orders of magnitude faster than BLAST. Bioinformatics *26*, 2460–2461.

ElMekawy, A., Hegab, H.M., and Pant, D. (2014). The near-future integration of microbial desalination cells with reverse osmosis technology. Energy Environ. Sci. *7*, 3921–3933.

Embree, M., Qiu, Y., Shieu, W., Nagarajan, H., O'Neil, R., Lovley, D., and Zengler, K. (2014). The Iron Stimulon and Fur Regulon of Geobacter sulfurreducens and Their Role in Energy Metabolism. Appl. Environ. Microbiol. *80*, 2918–2927.

Erable, B., Duţeanu, N.M., Ghangrekar, M.M., Dumas, C., and Scott, K. (2010). Application of electro-active biofilms. Biofouling *26*, 57–71.

Erable, B., Etcheverry, L., and Bergel, A. (2011). From microbial fuel cell (MFC) to microbial electrochemical snorkel (MES): maximizing chemical oxygen demand (COD) removal from wastewater. Biofouling *27*, 319–326.

Escapa, A., Mateos, R., Martínez, E.J., and Blanes, J. (2016). Microbial electrolysis cells: An emerging technology for wastewater treatment and energy recovery. From laboratory to pilot plant and beyond. Renew. Sustain. Energy Rev. *55*, 942–956.

Esteve-Núñez, A., Rothermich, M., Sharma, M., and Lovley, D. (2005). Growth of Geobacter sulfurreducens under nutrient-limiting conditions in continuous culture. Env. Microbiol *7*, 641–648.

Esteve-Núñez, A., Sosnik, J., Visconti, P., and Lovley, D.R. (2008). Fluorescent properties of c-type cytochromes reveal their potential role as an extracytoplasmic electron sink in Geobacter sulfurreducens. Environ. Microbiol. *10*, 497–505.

Esteve-Núñez, A., Busalmen, J.P., Berná, A., Gutiérrez-Garrán, C., and Feliu, J.M. (2011). Opportunities behind the unusual ability of geobacter sulfurreducens for exocellular respiration and electricity production. Energy Environ. Sci. *4*, 2066–2069.

Esteve-Núñez A. (2014). Long-term demonstration of a Bioelectrochemically constructed wetland for urban wastewater treatment. (Abu Dabi),.

Estevez-Canales, M., Kuzume, A., Borjas, Z., Füeg, M., Lovley, D., Wandlowsky, T., and Esteve-Nunez, A. (2014). A severe reduction in the Cytochrome C content of Geobacter sulfurreducens eliminates its capacity for extracellular electron transfer. Environ. Microbiol. Environ. Microbiol. Rep. *7*, 219–226.

Estevez-Canales, M., Berná, A., Borjas, Z., and Esteve-Núñez, A. (2015). Screen-Printed Electrodes: New Tools for Developing Microbial Electrochemistry at Microscale Level. Energies *8*, 13211–13221.

Estevez-Canales M. (2016). Novel bioelectrochemical approaches for exploring extracellular

electron transfer in Geobacter sulfurreducens. Alcalá de Henares.

Feng, Y., Wang, X., Logan, B.E., and Lee, H. (2008). Brewery wastewater treatment using air-cathode microbial fuel cells. Appl. Microbiol. Biotechnol. *78*, 873–880.

Fischer, F., Bastian, C., Happe, M., Mabillard, E., and Schmidt, N. (2011). Microbial fuel cell enables phosphate recovery from digested sewage sludge as struvite. Bioresour. Technol. *102*, 5824–5830.

Fiset, E., and Puig, S. (2015). Modified Carbon Electrodes: A New Approach for Bioelectrochemical Systems. J. Bioremediation Biodegrad. *6*.

Fradler, K.R., Kim, J.R., Boghani, H.C., Dinsdale, R.M., Guwy, A.J., and Premier, G.C. (2014a). The effect of internal capacitance on power quality and energy efficiency in a tubular microbial fuel cell. Process Biochem. *49*, 973–980.

Fradler, K.R., Michie, I., Dinsdale, R.M., Guwy, A.J., and Premier, G.C. (2014b). Augmenting Microbial Fuel Cell power by coupling with Supported Liquid Membrane permeation for zinc recovery. Water Res. *55*, 115–125.

Freguia, S., Rabaey, K., Yuan, Z., and Keller, J. (2007). Electron and Carbon Balances in Microbial Fuel Cells Reveal Temporary Bacterial Storage Behavior During Electricity Generation. Environ. Sci. Technol. *41*, 2915–2921.

Fux, C., and Siegrist, H. (2004). Nitrogen removal from sludge digester liquids by nitrification/denitrification or partial nitritation/anammox: environmental and economical considerations. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res. *50*, 19–26.

Gangadharan, P., and Nambi, I.M. (2015). Hexavalent chromium reduction and energy recovery by using dual-chambered microbial fuel cell. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res. *71*, 353–358.

Geelhoed, J.S., and Stams, A.J.M. (2011). Electricity-assisted biological hydrogen production from acetate by Geobacter sulfurreducens. Environ. Sci. Technol. *45*, 815–820.

Gescher, J., and Kappler, A. (2014). Microbial Metal Respiration: From Geochemistry to Potential Applications (Springer Science & Business Media).

Gimkiewicz, C., and Harnisch, F. (2013). Waste Water Derived Electroactive Microbial Biofilms: Growth, Maintenance, and Basic Characterization. J. Vis. Exp. *82*.

Ginestet, P. (2007). Comparative Evaluation of Sludge Reduction Routes (IWA Publishing).

González, M.P., Navarro, R., Saucedo, I., Avila, M., Revilla, J., and Bouchard, C. (2002). Purification of phosphoric acid solutions by reverse osmosis and nanofiltration. Desalination *147*, 315–320.

Gorby, Y.A., Yanina, S., McLean, J., Rosso, K.M., Moyles, D., Dohnalkova, A., Beveridge, T.J., and Chang, I.S. (2006). Electrically conductive bacterial nanowires produced by Shewanella oneidensis strain MR-1 and other microorganisms. PNAS *103*, 11358–11363.

Gregory, K.B., and Lovley, D.R. (2005). Remediation and recovery of uranium from contaminated subsurface environments with electrodes. Environ. Sci. Technol. *39*, 8943–8947.

Gregory, K.B., Bond, D.R., and Lovley, D.R. (2004). Graphite electrodes as electron donors for anaerobic respiration. Env. Microbiol *6*, 596–604.

Gupta, C.K., and Sathiyamoorthy, D. (1998). Fluid Bed Technology in Materials Processing (CRC Press).

Hanaki, K., Wantawin, C., and Ohgaki, S. (1990). Effects of the activity of heterotrophs on nitrification in a suspended-growth reactor. Water Res. *24*, 289–296.

He, Z., Wagner, N., Minteer, S.D., and Angenent, L.T. (2006). An Upflow Microbial Fuel Cell with an Interior Cathode: Assessment of the Internal Resistance by Impedance Spectroscopy. Environ. Sci. Technol. *40*, 5212–5217.

Hedlund, B.P., Gosink, J.J., and Staley, J.T. (1996). Phylogeny of Prosthecobacter, the fusiform caulobacters: members of a recently discovered division of the bacteria. Int. J. Syst. Bacteriol. *46*, 960–966.

Hees, W. van (1965). A Bacterial Methane Fuel Cell. J. Electrochem. Soc. *112*, 258–262.

Heijne, A.T., Liu, F., Weijden, R. van der, Weijma, J., Buisman, C.J.N., and Hamelers, H.V.M. (2010). Copper recovery combined with electricity production in a microbial fuel cell. Environ. Sci. Technol. *44*, 4376–4381.

Heijnen, J.J., Mulder, A., Enger, W., and Hoeks, F. (1989). Review on the application of anaerobic fluidized bed reactors in waste-water treatment. Chem. Eng. J. *41*, B37–B50.

Henze, M. (2008). Biological Wastewater Treatment: Principles, Modelling and Design (IWA Publishing).

Hochstrat, R., Wintgens, T., and Corvini, P. (2015). Immobilised Biocatalysts for Bioremediation of Groundwater and Wastewater (IWA Publishing).

Holmes, D.E., Finneran, K.T., O'Neil, R.A., and Lovley, D.R. (2002). Enrichment of Members of the Family Geobacteraceae Associated with Stimulation of Dissimilatory Metal Reduction in Uranium-Contaminated Aquifer Sediments. Appl. Environ. Microbiol. *68*, 2300–2306.

Holmes, D.E., Chaudhuri, S.K., Nevin, K.P., Mehta, T., Methe, B.A., Liu, A., Ward, J.E., Woodard, T.L., Webster, J., and Lovley, D.R. (2006). Microarray and genetic analysis of electron transfer to electrodes in Geobacter sulfurreducens. Env. Microbiol *8*, 1805–1815.

Huang, D.-Y., Zhou, S.-G., Chen, Q., Zhao, B., Yuan, Y., and Zhuang, L. (2011a). Enhanced anaerobic degradation of organic pollutants in a soil microbial fuel cell. Chem. Eng. J. *172*, 647–653.

Huang, L., Chai, X., Chen, G., and Logan, B.E. (2011b). Effect of Set Potential on Hexavalent Chromium Reduction and Electricity Generation from Biocathode Microbial Fuel Cells. Environ. Sci. Technol. *45*, 5025–5031.

Huggins, T., Wang, H., Kearns, J., Jenkins, P., and Ren, Z.J. (2014). Biochar as a sustainable electrode material for electricity production in microbial fuel cells. Bioresour. Technol. *157*, 114–119.

Huggins, T.M., Pietron, J.J., Wang, H., Ren, Z.J., and Biffinger, J.C. (2015). Graphitic biochar as a cathode electrocatalyst support for microbial fuel cells. Bioresour. Technol. *195*, 147–153.

Ichihashi, O., and Hirooka, K. (2012). Removal and recovery of phosphorus as struvite from swine wastewater using microbial fuel cell. Bioresour. Technol. *114*, 303–307.

Inoue, K., Qian, X., Morgado, L., Kim, B.C., Mester, T., Izallalen, M., Salgueiro, C.A., and Lovley, D.R. (2010). Purification and Characterization of OmcZ, an Outer-Surface, Octaheme c-Type Cytochrome Essential for Optimal Current Production by Geobacter sulfurreducens. Appl Env. Microbiol *76*, 3999–4007.

İrdemez, Ş., Demircioğlu, N., and Yildiz, Y.Ş. (2006). The effects of pH on phosphate removal from wastewater by electrocoagulation with iron plate electrodes. J. Hazard. Mater. *137*, 1231–1235.

Jacobson, K.S., Drew, D.M., and He, Z. (2011). Efficient salt removal in a continuously operated upflow microbial desalination cell with an air cathode. Bioresour. Technol. *102*, 376–380.

Jana, P.S., Katuri, K., Kavanagh, P., Kumar, A., and Leech, D. (2014). Charge transport in films of Geobacter sulfurreducens on graphite electrodes as a function of film thickness. Phys. Chem. Chem. Phys. *16*, 9039–9046.

Jensen, H.M., Albers, A.E., Malley, K.R., Londer, Y.Y., Cohen, B.E., Helms, B.A., Weigele, P., Groves, J.T., and Ajo-Franklin, C.M. (2010). Engineering of a synthetic electron conduit in living cells. Proc. Natl. Acad. Sci. *107*, 19213–19218.

Jiang, D., and Li, B. (2009). Granular activated carbon single-chamber microbial fuel cells (GAC-SCMFCs): A design suitable for large-scale wastewater treatment processes. Biochem. Eng. J. *47*, 31–37.

Jones, J.G. (2013). Advances in Microbial Ecology (Springer Science & Business Media).

Jourdin, L., Freguia, S., Flexer, V., and Keller, J. (2016). Bringing High-Rate, CO2-Based Microbial Electrosynthesis Closer to Practical Implementation through Improved Electrode Design and Operating Conditions. Environ. Sci. Technol. *50*, 1982–1989.

Jung, S., and Regan, J.M. (2007). Comparison of anode bacterial communities and performance in microbial fuel cells with different electron donors. Appl. Microbiol. Biotechnol. *77*, 393–402.

Kato, S. (2015). Biotechnological Aspects of Microbial Extracellular Electron Transfer. Microbes Environ. *30*, 133–139.

Kato, S., Hashimoto, K., and Watanabe, K. (2012). Microbial interspecies electron transfer via

electric currents through conductive minerals. Proc. Natl. Acad. Sci. *109*, 10042–10046.

Katuri, K.P., Kavanagh, P., Rengaraj, S., and Leech, D. (2010). Geobacter sulfurreducens biofilms developed under different growth conditions on glassy carbon electrodes: insights using cyclic voltammetry. Chem. Commun. *46*, 4758–4760.

Kelly, P.T., and He, Z. (2014a). Understanding the application niche of microbial fuel cells in a cheese wastewater treatment process. Bioresour. Technol. *157*, 154–160.

Kelly, P.T., and He, Z. (2014b). Nutrients removal and recovery in bioelectrochemical systems: a review. Bioresour. Technol. *153*, 351–360.

Kerrn-Jespersen, J.P., and Henze, M. (1993). Biological phosphorus uptake under anoxic and aerobic conditions. Water Res. *27*, 617–624.

Kiely, P.D., Cusick, R., Call, D.F., Selembo, P.A., Regan, J.M., and Logan, B.E. (2011). Anode microbial communities produced by changing from microbial fuel cell to microbial electrolysis cell operation using two different wastewaters. Bioresour. Technol. *102*, 388–394.

Kim, J.R., Zuo, Y., Regan, J.M., and Logan, B.E. (2008). Analysis of ammonia loss mechanisms in microbial fuel cells treating animal wastewater. Biotechnol. Bioeng. *99*, 1120–1127.

Kong, W., Guo, Q., Wang, X., and Yue, X. (2011). Electricity Generation from Wastewater Using an Anaerobic Fluidized Bed Microbial Fuel Cell. Ind. Eng. Chem. Res. *50*, 12225–12232.

Köroğlu, E.O., Özkaya, B., Denktaş, C., and Çakmakci, M. (2014). Electricity generating capacity and performance deterioration of a microbial fuel cell fed with beer brewery wastewater. J. Biosci. Bioeng. *118*, 672–678.

Kotloski, N.J., and Gralnick, J.A. (2013). Flavin electron shuttles dominate extracellular electron transfer by Shewanella oneidensis. mBio *4*.

Kramer, J., Soukiazian, S., Mahoney, S., and Hicks-Garner, J. (2012). Microbial fuel cell biofilm characterization with thermogravimetric analysis on bare and polyethyleneimine surface modified carbon foam anodes. J. Power Sources *210*, 122–128.

Kumar, G.G., Sarathi, V.G.S., and Nahm, K.S. (2013). Recent advances and challenges in the anode architecture and their modifications for the applications of microbial fuel cells. Biosens. Bioelectron. *43*, 461–475.

Kuntke, P., Śmiech, K.M., Bruning, H., Zeeman, G., Saakes, M., Sleutels, T.H.J.A., Hamelers, H.V.M., and Buisman, C.J.N. (2012). Ammonium recovery and energy production from urine by a microbial fuel cell. Water Res. *46*, 2627–2636.

Kuntke, P., Sleutels, T.H.J.A., Saakes, M., and Buisman, C.J.N. (2014). Hydrogen production and ammonium recovery from urine by a Microbial Electrolysis Cell. Int. J. Hydrog. Energy *39*, 4771–4778.

Larrosa, A., Lozano, L.J., Katuri, K.P., Head, I.M., Scott, K., and Godinez, C. (2009). On the repeatability and reproducibility of experimental two-chambered microbial fuel cells. Fuel *88*, 1852–1857.

Larsen, S., Nielsen, L.P., and Schramm, A. (2015). Cable bacteria associated with long-distance electron transport in New England salt marsh sediment. Environ. Microbiol. Rep. *7*, 175–179.

Leang, C., Qian, X., Mester, T., and Lovley, D.R. (2010). Alignment of the c-Type Cytochrome OmcS Along Pili of Geobacter sulfurreducens. Appl Env. Microbiol.

Leung, D.Y.C., and Xuan, J. (2015). Micro & Nano-Engineering of Fuel Cells (CRC Press).

Li, H., Chang, J., Liu, P., Fu, L., Ding, D., and Lu, Y. (2015). Direct interspecies electron transfer accelerates syntrophic oxidation of butyrate in paddy soil enrichments. Environ. Microbiol. *17*, 1533–1547.

Li, Q., Zhao, C., Chen, X., Wu, W., and Li, Y. (2009). Comparison of pulverized coal combustion in air and in O2/CO2 mixtures by thermo-gravimetric analysis. J. Anal. Appl. Pyrolysis *85*, 521–528.

Li, Z., Zhang, Y., LeDuc, P.R., and Gregory, K.B. (2011). Microbial electricity generation via microfluidic flow control. Biotechnol. Bioeng. *108*, 2061–2069.

Liang, B., Cheng, H., Van Nostrand, J.D., Ma, J., Yu, H., Kong, D., Liu, W., Ren, N., Wu, L., Wang, A., *et al.* (2014). Microbial community structure and function of Nitrobenzene reduction biocathode in response to carbon source switchover. Water Res. *54*, 137–148.

van Lier, J.B., Tilche, A., Ahring, B.K., Macarie, H., Moletta, R., Dohanyos, M., Pol, L.W., Lens, P., Verstraete, W., and Management Committee of the IWA Anaerobic Digestion Specialised Group (2001). New perspectives in anaerobic digestion. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res. *43*, 1–18.

Lim, S.J., Park, W., Kim, T.-H., and Shin, I.H. (2012). Swine wastewater treatment using a unique sequence of ion exchange membranes and bioelectrochemical system. Bioresour. Technol. *118*, 163–169.

Liu, H., and Logan, B.E. (2004). Electricity Generation Using an Air-Cathode Single Chamber Microbial Fuel Cell in the Presence and Absence of a Proton Exchange Membrane. Environ. Sci. Technol. *38*, 4040–4046.

Liu, Y., and Tay, J.H. (2001). Metabolic response of biofilm to shear stress in fixed-film culture. J. Appl. Microbiol. *90*, 337–342.

Liu, B., Lei, Y., and Li, B. (2014a). A batch-mode cube microbial fuel cell based “shock” biosensor for wastewater quality monitoring. Biosens. Bioelectron. *62*, 308–314.

Liu, D., Lei, L., Yang, B., Yu, Q., and Li, Z. (2013). Direct electron transfer from electrode to electrochemically active bacteria in a bioelectrochemical dechlorination system. Bioresour.

Technol. *148*, 9–14.

Liu, F., Rotaru, A.-E., Shrestha, P.M., Malvankar, N.S., Nevin, K.P., and Lovley, D.R. (2012). Promoting direct interspecies electron transfer with activated carbon. Energy Environ. Sci. *5*, 8982–8989.

Liu, J., Zhang, F., He, W., Zhang, X., Feng, Y., and Logan, B.E. (2014b). Intermittent contact of fluidized anode particles containing exoelectrogenic biofilms for continuous power generation in microbial fuel cells. J. Power Sources *261*, 278–284.

Lloyd, J.R., Leang, C., Hodges Myerson, A.L., Coppi, M.V., Cuifo, S., Methe, B., Sandler, S.J., and Lovley, D.R. (2003). Biochemical and genetic characterization of PpcA, a periplasmic c-type cytochrome in Geobacter sulfurreducens. Biochem J *369*, 153–161.

Logan, B.E. (2008). Microbial fuel cells (John Wiley & Sons).

Logan, B.E., and Rabaey, K. (2012). Conversion of Wastes into Bioelectricity and Chemicals by Using Microbial Electrochemical Technologies. Science *337*, 686–690.

Logan, B.E., and Regan, J.M. (2006). Microbial fuel cells--challenges and applications. Env. Sci Technol *40*, 5172–5180.

Logan, B.E., Call, D., Cheng, S., Hamelers, H.V.M., Sleutels, T.H.J.A., Jeremiasse, A.W., and Rozendal, R.A. (2008). Microbial Electrolysis Cells for High Yield Hydrogen Gas Production from Organic Matter. Environ. Sci. Technol. *42*, 8630–8640.

Lovley, D.R. (2006). Bug juice: harvesting electricity with microorganisms. Nat. Rev. Microbiol. *4*, 497–508.

Lovley, D.R. (2008). Extracellular electron transfer: wires, capacitors, iron lungs, and more. Geobiology *6*, 225–231.

Lovley, D.R. (2011). Reach out and touch someone: potential impact of DIET (direct interspecies energy transfer) on anaerobic biogeochemistry, bioremediation, and bioenergy. Rev. Environ. Sci. Biotechnol. *10*, 101–105.

Lovley, D.R. (2012). Electromicrobiology. Annu. Rev. Microbiol. *66*, 391–409.

Lovley, D.R., and Phillips, E.J. (1986). Organic Matter Mineralization with Reduction of Ferric Iron in Anaerobic Sediments. Appl Env. Microbiol *51*, 683–689.

Lovley, D.R., and Phillips, E.J.P. (1988). Novel Mode of Microbial Energy Metabolism: Organic Carbon Oxidation Coupled to Dissimilatory Reduction of Iron or Manganese. Appl. Environ. Microbiol. *54*, 1472–1480.

Lovley, D.R., Stolz, J.F., Nord, G.L., and Philips, E.J.P. (1987). Anaerobic production of magnetite by a dissimilatory iron-reducing microorganiof organic matter metabolism. Nature *330*, 252–254.

Lovley, D.R., Ueki, T., Zhang, T., Malvankar, N.S., Shrestha, P.M., Flanagan, K.A., Aklujkar,

M., Butler, J.E., Giloteaux, L., Rotaru, A.-E., *et al.* (2011). Geobacter: the microbe electric's physiology, ecology, and practical applications. Adv. Microb. Physiol. *59*, 1–100.

Lücker, S., Wagner, M., Maixner, F., Pelletier, E., Koch, H., Vacherie, B., Rattei, T., Damsté, J.S.S., Spieck, E., Le Paslier, D., *et al.* (2010). A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. Proc. Natl. Acad. Sci. U. S. A. *107*, 13479–13484.

Luo, H., Xu, P., and Ren, Z. (2012). Long-term performance and characterization of microbial desalination cells in treating domestic wastewater. Bioresour. Technol. *120*, 187–193.

Malaeb, L., Katuri, K.P., Logan, B.E., Maab, H., Nunes, S.P., and Saikaly, P.E. (2013). A Hybrid Microbial Fuel Cell Membrane Bioreactor with a Conductive Ultrafiltration Membrane Biocathode for Wastewater Treatment. Environ. Sci. Technol. *47*, 11821–11828.

Malvankar, N.S., Vargas, M., Nevin, K.P., Franks, A.E., Leang, C., Kim, B.-C., Inoue, K., Mester, T., Covalla, S.F., Johnson, J.P., *et al.* (2011). Tunable metallic-like conductivity in microbial nanowire networks. Nat. Nanotechnol. *6*, 573–579.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992). Phylogenetic Oligodeoxynucleotide Probes for the Major Subclasses of Proteobacteria: Problems and Solutions. Syst. Appl. Microbiol. *15*, 593–600.

Marshall, C.W., Ross, D.E., Fichot, E.B., Norman, R.S., and May, H.D. (2012). Electrosynthesis of Commodity Chemicals by an Autotrophic Microbial Community. Appl. Environ. Microbiol. *78*, 8412–8420.

Marshall, C.W., LaBelle, E.V., and May, H.D. (2013). Production of fuels and chemicals from waste by microbiomes. Curr. Opin. Biotechnol. *24*, 391–397.

Marsili, E., Sun, J., and Bond, D.R. (2010). Voltammetry and Growth Physiology of Geobacter sulfurreducens Biofilms as a Function of Growth Stage and Imposed Electrode Potential. Electroanalysis *22*, 865–874.

McEldowney, S., and Fletcher, M. (1987). Adhesion of bacteria from mixed cell suspension to solid surfaces. Arch. Microbiol. *148*, 57–62.

Meas, Y., Ramirez, J.A., Villalon, M.A., and Chapman, T.W. (2010). Industrial wastewaters treated by electrocoagulation. Electrochimica Acta *55*, 8165–8171.

Mehta, T., Coppi, M.V., Childers, S.E., and Lovley, D.R. (2005). Outer membrane c-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in Geobacter sulfurreducens. Appl Env. Microbiol *71*, 8634–8641.

Mergaert, J., Cnockaert, M.C., and Swings, J. (2003). Thermomonas fusca sp. nov. and Thermomonas brevis sp. nov., two mesophilic species isolated from a denitrification reactor with poly(epsilon-caprolactone) plastic granules as fixed bed, and emended description of the genus Thermomonas. Int. J. Syst. Evol. Microbiol. *53*, 1961–1966.

Methe, B.A., Nelson, K.E., Eisen, J.A., Paulsen, I.T., Nelson, W., Heidelberg, J.F., Wu, D., Wu, M., Ward, N., Beanan, M.J., *et al.* (2003). Genome of Geobacter sulfurreducens: metal reduction in subsurface environments. Science *302*, 1967–1969.

Miller, L.G., and Oremland, R.S. (2008). Electricity generation by anaerobic bacteria and anoxic sediments from hypersaline soda lakes. Extremophiles *12*, 837–848.

Min, B., and Logan, B.E. (2004). Continuous electricity generation from domestic wastewater and organic substrates in a flat plate microbial fuel cell. Env. Sci Technol *38*, 5809–5814.

Min, B., Kim, J., Oh, S., Regan, J.M., and Logan, B.E. (2005). Electricity generation from swine wastewater using microbial fuel cells. Water Res. *39*, 4961–4968.

Modin, O., and Gustavsson, D.J.I. (2014). Opportunities for microbial electrochemistry in municipal wastewater treatment--an overview. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res. *69*, 1359–1372.

Modin, O., Wang, X., Wu, X., Rauch, S., and Fedje, K.K. (2012). Bioelectrochemical recovery of Cu, Pb, Cd, and Zn from dilute solutions. J. Hazard. Mater. *235–236*, 291–297.

Mollah, M.Y.A., Schennach, R., Parga, J.R., and Cocke, D.L. (2001). Electrocoagulation (EC) — science and applications. J. Hazard. Mater. *84*, 29–41.

Mollah, M.Y.A., Morkovsky, P., Gomes, J.A.G., Kesmez, M., Parga, J., and Cocke, D.L. (2004). Fundamentals, present and future perspectives of electrocoagulation. J. Hazard. Mater. *114*, 199–210.

Mulder, A. (2003). The quest for sustainable nitrogen removal technologies. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res. *48*, 67–75.

Mulder, A., van de Graaf, A.A., Robertson, L.A., and Kuenen, J.G. (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. *16*, 177–183.

Nevin, K.P., and Lovley, D.R. (2002). Mechanisms for Fe(III) Oxide Reduction in Sedimentary Environments. Geomicrobiol. J. *19*, 141–159.

Nevin, K.P., Richter, H., Covalla, S.F., Johnson, J.P., Woodard, T.L., Orloff, A.L., Jia, H., Zhang, M., and Lovley, D.R. (2008). Power output and columbic efficiencies from biofilms of Geobacter sulfurreducens comparable to mixed community microbial fuel cells. Env. Microbiol *10*, 2505–2514.

Nevin, K.P., Woodard, T.L., Franks, A.E., Summers, Z.M., and Lovley, D.R. (2010). Microbial Electrosynthesis: Feeding Microbes Electricity To Convert Carbon Dioxide and Water to Multicarbon Extracellular Organic Compounds. mBio *1*, 103–110.

Nevin, K.P., Hensley, S.A., Franks, A.E., Summers, Z.M., Ou, J., Woodard, T.L., Snoeyenbos-West, O.L., and Lovley, D.R. (2011). Electrosynthesis of Organic Compounds from Carbon Dioxide Is Catalyzed by a Diversity of Acetogenic Microorganisms▿. Appl. Environ. Microbiol.

77, 2882–2886.

Nicolella, C., van Loosdrecht, M.C.M., and Heijnen, J.J. (2000). Wastewater treatment with particulate biofilm reactors. J. Biotechnol. *80*, 1–33.

Oremland, R.S., and Taylor, B.F. (1978). Sulfate reduction and methanogenesis in marine sediments. Geochim. Cosmochim. Acta *42*, 209–214.

Pant, D., Van Bogaert, G., Diels, L., and Vanbroekhoven, K. (2010). A review of the substrates used in microbial fuel cells (MFCs) for sustainable energy production. Bioresour. Technol. *101*, 1533–1543.

Patil, S.A., Arends, J.B.A., Vanwonterghem, I., van Meerbergen, J., Guo, K., Tyson, G.W., and Rabaey, K. (2015). Selective Enrichment Establishes a Stable Performing Community for Microbial Electrosynthesis of Acetate from CO_2. Environ. Sci. Technol. *49*, 8833–8843.

Peixoto, L., Min, B., Martins, G., Brito, A.G., Kroff, P., Parpot, P., Angelidaki, I., and Nogueira, R. (2011). In situ microbial fuel cell-based biosensor for organic carbon. Bioelectrochemistry *81*, 99–103.

Pfeffer, C., Larsen, S., Song, J., Dong, M., Besenbacher, F., Meyer, R.L., Kjeldsen, K.U., Schreiber, L., Gorby, Y.A., El-Naggar, M.Y., *et al.* (2012). Filamentous bacteria transport electrons over centimetre distances. Nature *491*, 218–221.

Pham, T.H., Rabaey, K., Aelterman, P., Clauwaert, P., De Schamphelaire, L., Boon, N., and Verstraete, W. (2006). Microbial Fuel Cells in Relation to Conventional Anaerobic Digestion Technology. Eng. Life Sci. *6*, 285–292.

Potter, M.C. (1911). Electrical Effects Accompanying the Decomposition of Organic Compounds. Proc. R. Soc. Lond. B Biol. Sci. *84*, 260–276.

Pous, N., Puig, S., Coma, M., Balaguer, M.D., and Colprim, J. (2013). Bioremediation of nitrate-polluted groundwater in a microbial fuel cell. J. Chem. Technol. Biotechnol. *88*, 1690–1696.

Pous, N., Puig, S., Dolors Balaguer, M., and Colprim, J. (2015). Cathode potential and anode electron donor evaluation for a suitable treatment of nitrate-contaminated groundwater in bioelectrochemical systems. Chem. Eng. J. *263*, 151–159.

Price, M.N., Dehal, P.S., and Arkin, A.P. (2010). FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. PLoS ONE *5*, e9490.

Puig, S., Serra, M., Vilar-Sanz, A., Cabré, M., Bañeras, L., Colprim, J., and Balaguer, M.D. (2011a). Autotrophic nitrite removal in the cathode of microbial fuel cells. Bioresour. Technol. *102*, 4462–4467.

Puig, S., Serra, M., Coma, M., Cabré, M., Dolors Balaguer, M., and Colprim, J. (2011b). Microbial fuel cell application in landfill leachate treatment. J. Hazard. Mater. *185*, 763–767.

Qin, B., Luo, H., Liu, G., Zhang, R., Chen, S., Hou, Y., and Luo, Y. (2012). Nickel ion removal

from wastewater using the microbial electrolysis cell. Bioresour. Technol. *121*, 458–461.

Rabaey, K., and Rozendal, R.A. (2010). Microbial electrosynthesis — revisiting the electrical route for microbial production. Nat. Rev. Microbiol. *8*, 706–716.

Rabaey, K., and Verstraete, W. (2005). Microbial fuel cells: novel biotechnology for energy generation. Trends Biotechnol. *23*, 291–298.

Rabaey, K., Bützer, S., Brown, S., Keller, J., and Rozendal, R.A. (2010). High Current Generation Coupled to Caustic Production Using a Lamellar Bioelectrochemical System. Environ. Sci. Technol. *44*, 4315–4321.

Rabaey, K., Girguis, P., and Nielsen, L.K. (2011). Metabolic and practical considerations on microbial electrosynthesis. Curr. Opin. Biotechnol. *22*, 371–377.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., and Lovley, D.R. (2005). Extracellular electron transfer via microbial nanowires. Nature *435*, 1098–1101.

Reguera, G., Nevin, K.P., Nicoll, J.S., Covalla, S.F., Woodard, T.L., and Lovley, D.R. (2006). Biofilm and Nanowire Production Leads to Increased Current in Geobacter sulfurreducens Fuel Cells. Appl. Environ. Microbiol. *72*, 7345–7348.

Reimers, C.E., Tender, L.M., Fertig, S., and Wang, W. (2001). Harvesting energy from the marine sediment--water interface. Environ. Sci. Technol. *35*, 192–195.

Ren, H., Lee, H.-S., and Chae, J. (2012). Miniaturizing microbial fuel cells for potential portable power sources: promises and challenges. Microfluid. Nanofluidics *13*, 353–381.

Ren, Z., Ward, T.E., and Regan, J.M. (2007). Electricity production from cellulose in a microbial fuel cell using a defined binary culture. Env. Sci Technol *41*, 4781–4786.

Richter, H., Lanthier, M., Nevin, K.P., and Lovley, D.R. (2007). Lack of Electricity Production by Pelobacter carbinolicus Indicates that the Capacity for Fe(III) Oxide Reduction Does Not Necessarily Confer Electron Transfer Ability to Fuel Cell Anodes. Appl. Environ. Microbiol. *73*, 5347–5353.

Richter, H., Nevin, K.P., Jia, H., Lowy, D.A., Lovley, D.R., and Tender, L.M. (2009). Cyclic voltammetry of biofilms of wild type and mutant Geobacter sulfurreducens on fuel cell anodes indicates possible roles of OmcB, OmcZ, type IV pili, and protons in extracellular electron transfer. Energy Environ. Sci. *2*, 506.

Richter, K., Schicklberger, M., and Gescher, J. (2012). Dissimilatory Reduction of Extracellular Electron Acceptors in Anaerobic Respiration. Appl. Environ. Microbiol. *78*, 913–921.

Rijnaarts, H.H.M., Norde, W., Bouwer, E.J., Lyklema, J., and Zehnder, A.J.B. (1993). Bacterial Adhesion under Static and Dynamic Conditions. Appl. Environ. Microbiol. *59*, 3255–3265.

Risgaard-Petersen, N., Kristiansen, M., Frederiksen, R.B., Dittmer, A.L., Bjerg, J.T., Trojan, D., Schreiber, L., Damgaard, L.R., Schramm, A., and Nielsen, L.P. (2015). Cable Bacteria in Freshwater Sediments. Appl. Environ. Microbiol. *81*, 6003–6011.

Rodenas Motos, P., ter Heijne, A., van der Weijden, R., Saakes, M., Buisman, C.J.N., and Sleutels, T.H.J.A. (2015). High rate copper and energy recovery in microbial fuel cells. Front. Microbiol. *6*.

Rodrigo, J., Boltes, K., and Esteve-Nuñez, A. (2014). Microbial-electrochemical bioremediation and detoxification of dibenzothiophene-polluted soil. Chemosphere *101*, 61–65.

Rodrigo Quejigo, J., Dörfler, U., Schroll, R., and Esteve-Núñez, A. (2016). Stimulating soil microorganisms for mineralizing the herbicide isoproturon by means of microbial electroremediating cells. Microb. Biotechnol. *9*, 369–380.

Rollefson, J.B., Stephen, C.S., Tien, M., and Bond, D.R. (2011). Identification of an Extracellular Polysaccharide Network Essential for Cytochrome Anchoring and Biofilm Formation in Geobacter sulfurreducens. J. Bacteriol. *193*, 1023–1033.

Rosenbaum, M.A., and Franks, A.E. (2013). Microbial catalysis in bioelectrochemical technologies: status quo, challenges and perspectives. Appl. Microbiol. Biotechnol. *98*, 509–518.

Rotaru, A.-E., Shrestha, P.M., Liu, F., Ueki, T., Nevin, K., Summers, Z.M., and Lovley, D.R. (2012). Interspecies electron transfer via hydrogen and formate rather than direct electrical connections in cocultures of Pelobacter carbinolicus and Geobacter sulfurreducens. Appl. Environ. Microbiol. *78*, 7645–7651.

Rotaru, A.-E., Shrestha, P.M., Liu, F., Markovaite, B., Chen, S., Nevin, K., and Lovley, D. (2014). Direct Interspecies Electron Transfer Between Geobacter metallireducens and Methanosarcina barkeri. Appl. Environ. Microbiol. *80*, 4599–4605.

Rotaru, A.-E., Woodard, T.L., Nevin, K.P., and Lovley, D.R. (2015). Link between capacity for current production and syntrophic growth in Geobacter species. Front. Microbiol. *6*, 744.

Rozendal, R.A., Hamelers, H.V.M., Rabaey, K., Keller, J., and Buisman, C.J.N. (2008). Towards practical implementation of bioelectrochemical wastewater treatment. Trends Biotechnol. *26*, 450–459.

Rozendal, R.A., Leone, E., Keller, J., and Rabaey, K. (2009). Efficient hydrogen peroxide generation from organic matter in a bioelectrochemical system. Electrochem. Commun. *11*, 1752–1755.

Saeed, H.M., Husseini, G.A., Yousef, S., Saif, J., Al-Asheh, S., Abu Fara, A., Azzam, S., Khawaga, R., and Aidan, A. (2015). Microbial desalination cell technology: A review and a case study. Desalination *359*, 1–13.

Santoro, C., Guilizzoni, M., Correa Baena, J.P., Pasaogullari, U., Casalegno, A., Li, B., Babanova, S., Artyushkova, K., and Atanassov, P. (2014). The effects of carbon electrode surface properties on bacteria attachment and start up time of microbial fuel cells. Carbon *67*, 128–139.

Sayess, R.R., Saikaly, P.E., El-Fadel, M., Li, D., and Semerjian, L. (2013). Reactor

performance in terms of COD and nitrogen removal and bacterial community structure of a three-stage rotating bioelectrochemical contactor. Water Res. *47*, 881–894.

Schauer, R., Risgaard-Petersen, N., Kjeldsen, K.U., Tataru Bjerg, J.J., Jørgensen, B.B., Schramm, A., and Nielsen, L.P. (2014). Succession of cable bacteria and electric currents in marine sediment. ISME J. *8*, 1314–1322.

Schröder, U. (2007). Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency. Phys. Chem. Chem. Phys. *9*, 2619–2629.

Schrott, G.D., Bonanni, P.S., Robuschi, L., Esteve-Nuñez, A., and Busalmen, J.P. (2011). Electrochemical insight into the mechanism of electron transport in biofilms of Geobacter sulfurreducens. Electrochimica Acta *56*, 10791–10795.

Schrott, G.D., Ordoñez, M.V., Robuschi, L., and Busalmen, J.P. (2014). Physiological stratification in electricity-producing biofilms of Geobacter sulfurreducens. ChemSusChem *7*, 598–603.

Scott, K., and Yu, E.H. (2015). Microbial Electrochemical and Fuel Cells: Fundamentals and Applications (Woodhead Publishing).

Shannon, P. (2003). Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. *13*, 2498–2504.

Shao, Y., Chung, B.S., Lee, S.S., Park, W., Lee, S.-S., and Jeon, C.O. (2009). Zoogloea caeni sp. nov., a floc-forming bacterium isolated from activated sludge. Int. J. Syst. Evol. Microbiol. *59*, 526–530.

Shrestha, P.M., and Rotaru, A.-E. (2014). Plugging in or going wireless: strategies for interspecies electron transfer. Front. Microbiol. *5*, 237.

Simate, G.S., Cluett, J., Iyuke, S.E., Musapatika, E.T., Ndlovu, S., Walubita, L.F., and Alvarez, A.E. (2011). The treatment of brewery wastewater for reuse: State of the art. Desalination *273*, 235–247.

Sleutels, T.H.J.A., Ter Heijne, A., Buisman, C.J.N., and Hamelers, H.V.M. (2012). Bioelectrochemical systems: an outlook for practical applications. ChemSusChem *5*, 1012–1019.

Sleutels, T.H.J.A., Molenaar, S.D., Heijne, A.T., and Buisman, C.J.N. (2016). Low Substrate Loading Limits Methanogenesis and Leads to High Coulombic Efficiency in Bioelectrochemical Systems. Microorganisms *4*, 7.

Sokatch, J.R. (2014). Bacterial Physiology and Metabolism (Academic Press).

Speers, A.M., and Reguera, G. (2012). Electron donors supporting growth and electroactivity of Geobacter sulfurreducens anode biofilms. Appl. Environ. Microbiol. *78*, 437–444.

Stahl, D.A., Amann, R., and Stahl, D.A. (1991). Development and application of nucleic acid probes.

Steinbusch, K.J.J., Hamelers, H.V.M., Schaap, J.D., Kampman, C., and Buisman, C.J.N. (2010). Bioelectrochemical Ethanol Production through Mediated Acetate Reduction by Mixed Cultures. Environ. Sci. Technol. *44*, 513–517.

Stookey, L.L. (1970). Ferrozine---a new spectrophotometric reagent for iron. Anal. Chem. *42*, 779–781.

Strycharz, S.M., Woodard, T.L., Johnson, J.P., Nevin, K.P., Sanford, R.A., Loffler, F.E., and Lovley, D.R. (2008). Graphite electrode as a sole electron donor for reductive dechlorination of tetrachlorethene by Geobacter lovleyi. Appl Env. Microbiol *74*, 5943–5947.

Summers, Z.M., Fogarty, H.E., Leang, C., Franks, A.E., Malvankar, N.S., and Lovley, D.R. (2010). Direct Exchange of Electrons Within Aggregates of an Evolved Syntrophic Coculture of Anaerobic Bacteria. Science *330*, 1413–1415.

Tang, Y.J., Chakraborty, R., Martín, H.G., Chu, J., Hazen, T.C., and Keasling, J.D. (2007). Flux Analysis of Central Metabolic Pathways in Geobacter metallireducens during Reduction of Soluble Fe(III)-Nitrilotriacetic Acid. Appl. Environ. Microbiol. *73*, 3859–3864.

Tchobanoglous, G., and Burton, F.L. (1991). Wastewater engineering: treatment, disposal, and reuse (McGraw-Hill).

Tian, Y., He, W., Zhu, X., Yang, W., Ren, N., and Logan, B.E. (2016). Energy efficient electrocoagulation using an air-breathing cathode to remove nutrients from wastewater. Chem. Eng. J. *292*, 308–314.

Tong, Y., and He, Z. (2013). Nitrate removal from groundwater driven by electricity generation and heterotrophic denitrification in a bioelectrochemical system. J. Hazard. Mater. *262*, 614–619.

Torres, C.I., Kato Marcus, A., and Rittmann, B.E. (2008). Proton transport inside the biofilm limits electrical current generation by anode-respiring bacteria. Biotechnol. Bioeng. *100*, 872–881.

Ueki, T., Nevin, K.P., Woodard, T.L., and Lovley, D.R. (2014). Converting carbon dioxide to butyrate with an engineered strain of Clostridium ljungdahlii. mBio *5*, e01636–01614.

Uria, N., Abramova, N., Bratov, A., Muñoz-Pascual, F.-X., and Baldrich, E. (2016). Miniaturized metal oxide pH sensors for bacteria detection. Talanta *147*, 364–369.

Valero, D., Ortiz, J.M., García, V., Expósito, E., Montiel, V., and Aldaz, A. (2011). Electrocoagulation of wastewater from almond industry. Chemosphere *84*, 1290–1295.

Van Loosdrecht, M.C.M., and Jetten, M.S.M. (1998). Microbiological conversions in nitrogen removal. Water Sci. Technol. *38*, 1–7.

Verea, L., Savadogo, O., Verde, A., Campos, J., Ginez, F., and Sebastian, P.J. (2014). Performance of a microbial electrolysis cell (MEC) for hydrogen production with a new process for the biofilm formation. Int. J. Hydrog. Energy *39*, 8938–8946.

Villano, M., Aulenta, F., Ciucci, C., Ferri, T., Giuliano, A., and Majone, M. (2010). Bioelectrochemical reduction of CO(2) to CH(4) via direct and indirect extracellular electron transfer by a hydrogenophilic methanogenic culture. Bioresour. Technol. *101*, 3085–3090.

Virdis, B., Rabaey, K., Yuan, Z., and Keller, J. (2008). Microbial fuel cells for simultaneous carbon and nitrogen removal. Water Res. *42*, 3013–3024.

Virdis, B., Rabaey, K., Rozendal, R.A., Yuan, Z., and Keller, J. (2010). Simultaneous nitrification, denitrification and carbon removal in microbial fuel cells. Water Res. *44*, 2970–2980.

Virdis, B., Read, S.T., Rabaey, K., Rozendal, R.A., Yuan, Z., and Keller, J. (2011). Biofilm stratification during simultaneous nitrification and denitrification (SND) at a biocathode. Bioresour. Technol. *102*, 334–341.

Virdis, B., Millo, D., Donose, B.C., and Batstone, D.J. (2014). Real-Time Measurements of the Redox States of c-Type Cytochromes in Electroactive Biofilms: A Confocal Resonance Raman Microscopy Study. PLoS ONE *9*.

Voordeckers, J.W., Kim, B.-C., Izallalen, M., and Lovley, D.R. (2010). Role of Geobacter sulfurreducens outer surface c-type cytochromes in reduction of soil humic acid and anthraquinone-2,6-disulfonate. Appl. Environ. Microbiol. *76*, 2371–2375.

Wang, H., and Ren, Z.J. (2013). A comprehensive review of microbial electrochemical systems as a platform technology. Biotechnol. Adv. *31*, 1796–1807.

Wang, H., and Ren, Z.J. (2014). Bioelectrochemical metal recovery from wastewater: A review. Water Res. *66*, 219–232.

Wang, X., Feng, Y.J., and Lee, H. (2008). Electricity production from beer brewery wastewater using single chamber microbial fuel cell. Water Sci Technol *57*, 1117–1121.

Wang, Y., Zhang, Y., Wang, J., and Meng, L. (2009). Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. Biomass Bioenergy *33*, 848–853.

Ward, N.L., Challacombe, J.F., Janssen, P.H., Henrissat, B., Coutinho, P.M., Wu, M., Xie, G., Haft, D.H., Sait, M., Badger, J., *et al.* (2009). Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils. Appl. Environ. Microbiol. *75*, 2046–2056.

Watanabe, T., Sumida, H., DO, N.M., Yano, K., Asakawa, S., and Kimura, M. (2013). Bacterial consortia in iron-deposited colonies formed on paddy soil surface under microaerobic conditions. Soil Sci. Plant Nutr. *59*, 337–346.

Wei, Y., Van Houten, R.T., Borger, A.R., Eikelboom, D.H., and Fan, Y. (2003). Minimization of excess sludge production for biological wastewater treatment. Water Res. *37*, 4453–4467.

Xie, C.-H., and Yokota, A. (2005). Reclassification of Alcaligenes latus strains IAM 12599T and

IAM 12664 and Pseudomonas saccharophila as Azohydromonas lata gen. nov., comb. nov., Azohydromonas australica sp. nov. and Pelomonas saccharophila gen. nov., comb. nov., respectively. Int. J. Syst. Evol. Microbiol. *55*, 2419–2425.

Xu, L., Zhao, Y., Doherty, L., Hu, Y., and Hao, X. (2016). The integrated processes for wastewater treatment based on the principle of microbial fuel cells: A review. Crit. Rev. Environ. Sci. Technol. *46*, 60–91.

Yan, H., Saito, T., and Regan, J.M. (2012a). Nitrogen removal in a single-chamber microbial fuel cell with nitrifying biofilm enriched at the air cathode. Water Res. *46*, 2215–2224.

Yan, Z., Song, N., Cai, H., Tay, J.-H., and Jiang, H. (2012b). Enhanced degradation of phenanthrene and pyrene in freshwater sediments by combined employment of sediment microbial fuel cell and amorphous ferric hydroxide. J. Hazard. Mater. *199-200*, 217–225.

Yu, H., Feng, C., Liu, X., Yi, X., Ren, Y., and Wei, C. (2016). Enhanced anaerobic dechlorination of polychlorinated biphenyl in sediments by bioanode stimulation. Environ. Pollut. Barking Essex 1987 *211*, 81–89.

Yu, J., Park, Y., Kim, B., and Lee, T. (2015). Power densities and microbial communities of brewery wastewater-fed microbial fuel cells according to the initial substrates. Bioprocess Biosyst. Eng. *38*, 85–92.

Zhang, B., Feng, C., Ni, J., Zhang, J., and Huang, W. (2012). Simultaneous reduction of vanadium (V) and chromium (VI) with enhanced energy recovery based on microbial fuel cell technology. J. Power Sources *204*, 34–39.

Zhang, T., Gannon, S.M., Nevin, K.P., Franks, A.E., and Lovley, D.R. (2010). Stimulating the anaerobic degradation of aromatic hydrocarbons in contaminated sediments by providing an electrode as the electron acceptor. Env. Microbiol.

Zhang, Y., Noori, J.S., and Angelidaki, I. (2011). Simultaneous organic carbon, nutrients removal and energy production in a photomicrobial fuel cell (PFC). Energy Environ. Sci. *4*, 4340–4346.

Zhuang, L., Zhou, S., Li, Y., and Yuan, Y. (2010). Enhanced performance of air-cathode two-chamber microbial fuel cells with high-pH anode and low-pH cathode. Bioresour. Technol. *101*, 3514–3519.

Zhuang, L., Yuan, Y., Wang, Y., and Zhou, S. (2012a). Long-term evaluation of a 10-liter serpentine-type microbial fuel cell stack treating brewery wastewater. Bioresour. Technol. *123*, 406–412.

Zhuang, L., Zheng, Y., Zhou, S., Yuan, Y., Yuan, H., and Chen, Y. (2012b). Scalable microbial fuel cell (MFC) stack for continuous real wastewater treatment. Bioresour. Technol. *106*, 82–88.

ANNEX

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Abbreviations

∆E	Difference of potential
1C	First chamber
2C	Second chamber
AD	Anaerobic digestion
AE	Auxiliar or counter electrode
AEM	Anion exchange membrane
AOB	Ammonium oxidizing bacteria
BES	Bioelectrochemical system
BOD	Biological oxygen demand
CE	Coulombic efficiency
CEM	Cation exchange membrane
COD	Chemical oxygen demand
DAPI	4',6-diamidino-2-phenylindole
DEET	Direct extracellular electron transfer
DIET	Direct interspecies electron transfer
DO	Dissolved oxygen
dw	Dry weight
E_{anode}	Anode potential
EC	Electrocoagulation
$E_{cathode}$	Cathode potential
ED	Electron donor
EET	Extracellular electron transfer
EPS	extracellular polymeric substances
HRT	Hydraulic retention time
IET	Interspecies electron transfer
j	Current density

M-FBR	Microbial fluidized bed reactor
MDC	Microbial desalination cell
ME-FBR	Microbial electrochemical fluidized bed reactor
MEC	Microbial electrolysis cell
MEET	Mediated extracellular electron transfer
MERC	Microbial electroremediating cell
MES	Microbial electrosynthesis cell
MESC	Microbial electrolysis struvite-precipitation cell
MET	Microbial electrochemical technology
MFC	Microbial fuel cell
N	Nitrogen
NOB	Nitrite oxidizing bacteria
OCP	Open circuit potential
OD	Optical density
OLR	Organic loading rate
ORR	Organic removal rate
P	Phosphorous
PBS	Phosphate buffer solution
RE	Reference electrode
RT	Reaction time
SDS	Sodium dodecyl sulphate
SDTA	Simulteneous differential thermal analysis
SEM	Scanning electron microscopy
TC	Treatment capacity
TCC	Total cathodic compartment
TEA	Terminal electron acceptor
TG	Thermogravimetry

TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorous
TS	Total solids
TSS	Total suspended solids
WE	Working electrode
WWT	Wastewater treatment
WWTP	Wastewater treatment plant

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